Screening the Ubiquitination Components Essential for Cardiomyocyte Proliferation and Cardiac Regeneration

Thesis

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Screening the ubiquitination components essential for cardiomyocyte proliferation and cardiac regeneration

Diem My Vu

A Thesis submitted in fulfillment of the requirements of the Faculty of Life Sciences of the Open University (UK) for the Degree of Doctor of Philosophy

Director of Studies:
Professor Mauro Giacca

External Supervisor:
Professor Thierry Pedrazzini

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ABSTRACT

For several years, the inability to replace the lost myocardium has been studied intensively to define the mechanisms that restrict the regenerative capability of the adult heart. Multiple evidence have pointed out that the turnover of postnatal mammalian cardiomyocytes is affected by different crucial pathways. However, a thorough understanding of the intrinsic molecular mechanisms that regulate this process is still far from being complete.

It has been more than a decade since various laboratories initialised the study of ubiquitination processes in the heart, reaching the unanimous conclusion that it plays a critical role in manipulating virtually all heart functions. However, there is fragmentary information about the molecular function of this system on the regulation of cardiomyocyte proliferation and even less on how this could be exploited for therapeutic purposes. Hence, expanding our knowledge on the ubiquitination might devise new strategies for cardiac regeneration.

In this work, we present the identification of ubiquitination factors that are essential for cardiomyocyte replication, which was identified by harnessing the High Throughput Screening approach. An RNAi-based screening was performed, in which approximately 600 ubiquitination factors were silenced individually in primary neonatal cardiomyocytes. After that, the top siRNAs inhibiting proliferation were selected and investigated further to validate their functions *in vitro*. The validation identified UBE2G1, an E2 conjugation enzyme, as the most effective factor induced cardiomyocyte proliferation. The depletion of UBE2G1 not only suppressed cell cycle progression but also stimulated hypertrophy and counteracted the effects of pro-proliferative miRNAs. Administration of AAV9-UBE2G1 in neonatal mice promoted the cycling of cardiomyocytes while it preserved the heart function in adult mice at the early time points after myocardial infarction. Activation of GSK-3β, ERK1/2, and STAT3 signalling pathways

correlated with UBE2G1 activity, whereas its interacting partners remained to be identified. In summary, we have revealed the implicit potential of ubiquitination factors and highlighted their promising therapeutic use in a heart regeneration scenario.

1. INTRODUCTION

1.1 Cardiovascular diseases

1.1.1 The demand for cardiovascular disease studies

Cardiovascular diseases (CVDs) have been placed as the top cause of death worldwide, accounting for over 30% of all deaths, with a total of 422.7 million cases globally in 2015 (Roth et al. 2017). A few decades ago, we observed a phenomenal decline of CVD mortality in nearly all regions, with the massive reduction reached 70% in the Netherland (Mensah et al. 2017). Achievements eased our concerns and rapid progress of treatment and prevention that anticipated the control of CVDs mortality. Suddenly, we observe a gradual acceleration of CVDs death cases in both genders throughout the world (Lopez and Adair 2019). This information indicates much works remain to be done in treating heart disease before reaching our ultimate goal of reducing its burden.

Poor prognosis remains a prominent characteristic of CVDs. After 35 years of medicine development, we have raised 10-year survival of heart failure (HF) patients from 20% to 26.2%, and minimal progress was gained in treating the end-stage outcome (Mahmood et al. 2014; Taylor et al. 2019). In terms of CVD epidemiology, its utility was questioned in previous periods and even more recently (Vasan and Benjamin 2016). Approximately 15-20% of myocardial infarction (MI) patients had none of the classical risk factors, while more than 50% of HF patients showed average ejection fraction (EF) at the time of diagnosis (Ruwanpathirana et al. 2015). For 70 years of studying, the Framingham Heart Study provides excellent insights into the risk factor classification and genetic contribution to heart disease development although leaving behind mysterious targets for treatment therapy (Andersson et al. 2019). The variances of populations and lifestyle factors make it more complications to predict and intervene beforehand. Therefore, the cutting edges of biomedical research are desired more than ever to propel the future of medicine. We are waiting for the leap of

cardiovascular medicine through the rapid advances of big data and functional omics to overcome the current challenges.

1.1.2 Mechanisms of heart development

Heart development has been studied intensively over a few last decades to better understand the molecular mechanisms driving this process. And this direction is considered as a central cornerstone to navigate treatment therapy. Besides the solid knowledge of foetal cardiomyocyte specification and the basic findings in the neonatal and adult heart, we still lack much information about the postnatal development of cardiomyocytes. To regenerate efficiently the heart after cardiac injuries, replacing cardiomyocytes must undergo the exquisite maturation to functional synchronisation with pre-existing myocardium. Thus, we will discuss the details of the cardiomyocyte maturation in this part.

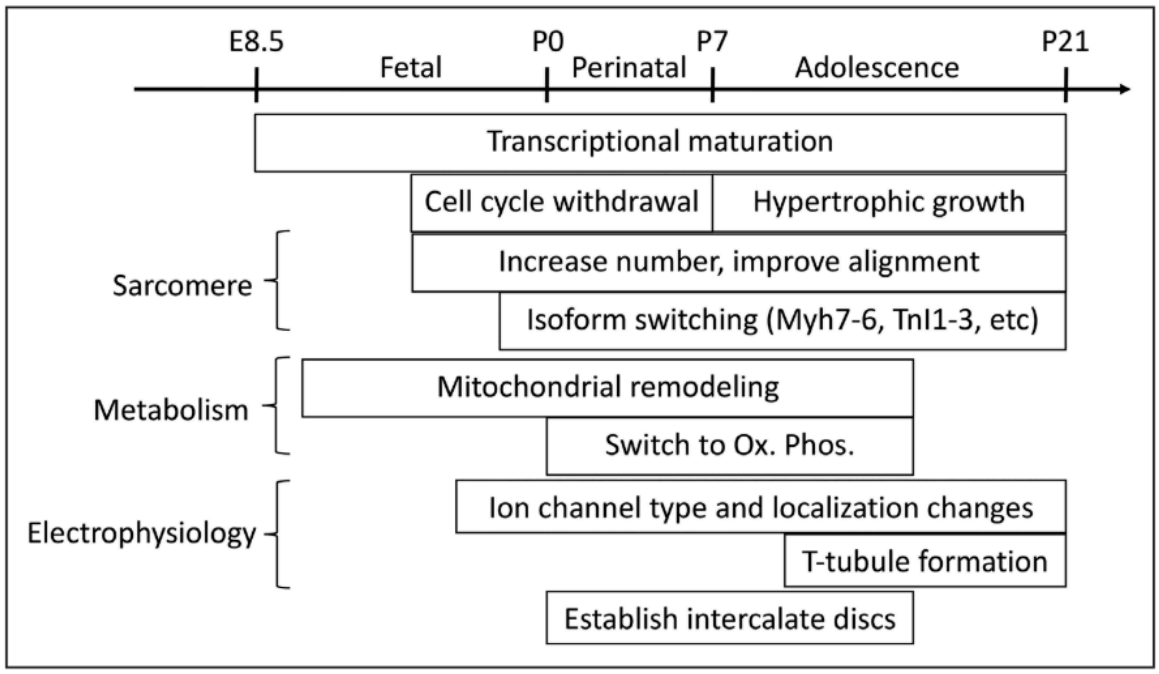

Figure 1.1: Representative cellular events during cardiomyocyte maturation (Galdos et al. 2017). The maturation process starts early at the embryonic stage and continues at the postnatal stage with modifications in gene expression, cell cycling, cardiomyocyte structural arrangement, metabolism switching and the formation of a mature electrophysiology compartment.

Myofibrils. Cardiac myocytes underwent drastic modifications of the morphology during the development. Early after birth, mitosis quiescence arises, and the cells grow massively in volume. The cell dimension extents 10-20 times, and they become rod-shaped with specific ultrastructures (Mollova et al. 2013; Scuderi and Butcher 2017). Cardiomyocyte ultrastructure consists of adjacent myofibril subunits, which serve as the contractile apparatus. During the maturation, several myofibrillar proteins switch their isoform expressions such as titin (TTN-N2BA to TTN-N2B), myosin (MYH6 to MYH7 in human, MYL7 to MYL2), and troponin (TNNI1 to TNNI3). Compared with the foetal stage, these modifications result in better alignment of the myofibrillar structure while enhancing the membrane capacitance following the enlargement of cell surface area. These changes also provide a greater velocity of signal electrical transmission and increase the contraction force (Spach et al. 2004; Wiegerinck et al. 2009). However, the actual molecular mechanisms driving this process remain elusive.

Electrophysiology. Cardiomyocyte synchronous beating is controlled tightly by electrical impulses and oscillations. Briefly, sodium and calcium channels depolarise the cells, outward potassium channels repolarise portions of the membrane, calcium ion travels inward to excite the contraction while potassium ion is pumped outward to drive the membrane resting, and the inward rectifying potassium channels maintain the resting phase (van den Heuvel et al. 2014; Liu et al. 2016a). Since multiple ion channels participate in this reaction, the electrophysiological machinery evolves progressively through different heart development stages. Cariomyocyte maturation correlates with an increase of ventricular channel and a decrease of automated channel presence (KCNJ2 and HCN4, respectively); adult myocytes exhibit low automaticity until triggered by neighboring cells, with less resting membrane potential, and longer action potential duration (Buchanan Jr et al. 1985; Liu et al. 2016a). The intercalated disc is a unique feature of cardiac cell-cell connections, which forms shortly after birth and involves the assembly of various cell adhesion

molecules to allow rapid electrical propagation (Noorman et al. 2009; Vermij et al. 2017).

Calcium handling. Calcium ion plays a prominent role in regulating cardiomyocyte gene expression, differentiation, and development (Louch et al. 2015). Cardiomyocyte maturation correlates with increased significantly the expression of Ca^{2+} handling molecules such as ryanodine receptors (RyRs) and sarcoplasmic-endoplasmic reticulum calcium ATPase 2a (SERCA2a) (Ruan et al. 2016; Huang et al. 2020). The structural components show the gradual loss of T-type channels, whereas T-tubules containing L-type channels develop postnatally (Louch et al. 2006). These modifications accelerate Ca^{2+} signalling dynamics to compensate for the relatively low Ca^{2+} intracellular concentration. Of note, around 30% of total heart energy is dedicated to Ca^{2+} trafficking, and thus the effective regulation of the flux assists heart function (Smith and Eisner 2019). The defects in Ca^{2+} handling and embryonic reversal of its compartments are commonly observed in heart failure and many kinds of heart diseases (Gomez et al. 2001; Lenaerts et al. 2009).

Metabolism. To meet the high demand for energy of the postnatal heart, cardiac myocytes progressively switch from glycolysis to oxidative metabolism. This metabolic shift was connected to the induction of the Peroxisome Proliferator Activated Receptor α (PPARα) and its co-activator PGC-1α in several studies (Guo and Pu 2020). In parallel, the upregulation of genes encoding oxidative metabolism components and downregulation of glycolytic genes were also observed (Uosaki et al. 2015; Malandraki-Miller et al. 2018). Nevertheless, the actual mechanisms regulating this transition are mostly unknown. Notably, mitochondria appear to play a crucial role in this process. Mature mitochondria contain dense cristae, the folding layer of the inner membrane, which elevates cellular respiration capacity. They also grow in size, number, reshape to oval and occupy at least 30% of the cell volume eventually (Piquereau et al. 2013; Scuderi and Butcher 2017). Mitochondria are distributed along the myofibrils to support

the contraction and ion exchange. Dysfunction of mitochondria not only results in energy deprivation but is also linked directly to cardiomyocyte damage, promoting heart failure progression or dilated cardiomyopathy (Brown et al. 2017a; Zhao et al. 2019).

Cell cycle. The heart undergoes cell cycle quiescence after one week in mice and slowly in the first year of humans postnatally. The decrease of cyclins and cyclin-dependent kinases (CDKs) expression after birth have been reported in many studies, concurrently with increased expression of CDK inhibitors (CKIs) during development (Tzahor and Poss 2017; Hashmi and Ahmad 2019). Mature cardiomyocytes of most mammalian species are multinucleated. Mouse, rat and rabbit have binucleated hearts while the multinucleation predominates in porcine hearts (Beinlich et al. 1995; Bensley et al. 2016). The distribution of the binucleated cell population was highlighted, by which roughly 80% of ventricular cardiomyocytes were binucleated in contrast with 14% of atrial myocytes in adult mouse heart (Raulf et al. 2015). Human adult cardiomyocytes are mostly mononuclear but polyploid due to insufficient karyokinesis was noted from the second decade of life (Mollova et al. 2013; Bergmann et al. 2015). Hypertension was reported to trigger cardiomyocyte ploidy, and a similar observation was noticed in the infarcted heart (Vliegen et al. 1995; Meckert et al. 2005). Polyploidisation has been associated negatively with heart regeneration, although its actual role remains enigmatic. Uncommon speculation suggests that DNA damage could be dodged through this process to prevent apoptosis (Gan et al. 2019; Derks and Bergmann 2020).

Together, understanding cardiomyocyte natural development processes will provide valuable insights that could be the source of action towards heart regeneration therapy.

1.1.3 Cardiac treatment strategies

Despite enthusiasm and efforts in many heart disease treatment trials, we are still seeking an effective strategy to regenerate sufficiently human heart after the injury (Hashimoto et al. 2018). Transplantation remains the

sole solution once patients develop end-stage failure. However, the demand for transplantation is met by 10%, due to the lack of donors worldwide and the surgical complexity (Trulock et al. 2007; White et al. 2018).

Myocardial injury is followed by sequential cellular processes to preserve heart function. In brief, an acute inflammatory response promotes immune cell infiltration to remove the wound of tissue debris, followed by myofibroblast proliferation, extracellular matrix deposition, and scar formation (Liehn et al. 2011; Prabhu and Frangogiannis 2016). The loss of tissue and the scar formation promote adverse left ventricular remodelling, leading to patient's heart failure.

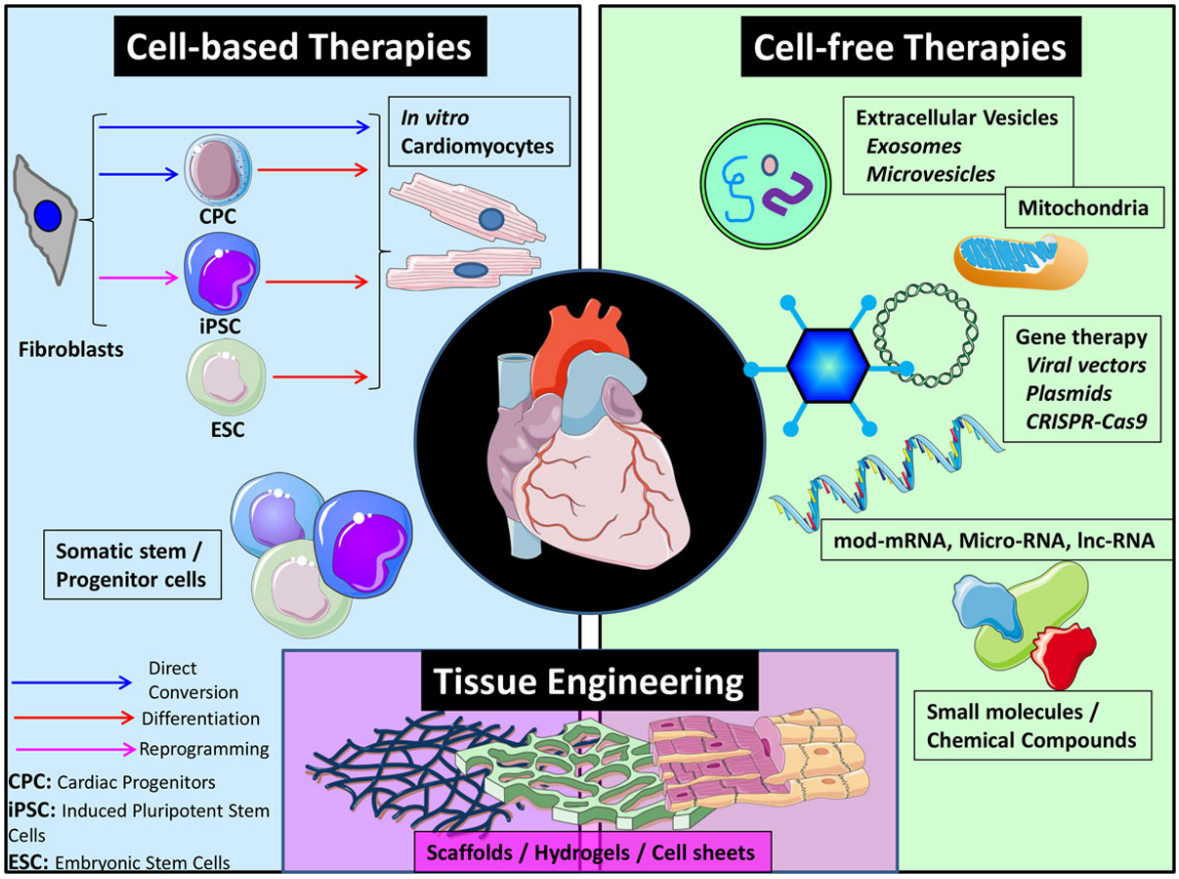

Figure 1.2: Current approaches for heart regeneration (Grigorian Shamagian et al. 2019). These include transplantation of cardiomyocytes generated from different sources (embryonic cells, iPS cells, transdifferentiation from fibroblasts), the delivery of various stimulators that enhance the endogenous regenerative capacity or the engraftment of engineered muscle tissue to support and promote the recovery of

the heart.

Heart regeneration might be accomplished through numerous strategies, mainly centralised in these directions: transplantation of pluripotent cell-derived cardiomyocytes, direct reprogramming of non-myocytes to cardiac fate, or stimulation of the innate proliferative capacity of cardiomyocytes (Hashimoto et al. 2018; Sadek and Olson 2020).

1.1.3.1 Cell-based therapies (transplantation)

Mesenchymal stromal cells (MSCs). Transplantation of MSCs seemed to hold a tremendous promise for heart regeneration among diverse bone marrow-derived cells (Hare et al. 2012; Menasché 2018). The benefits of using MSCs as a replacement source counts on their low immunogenicity and strong paracrine effects compared with other cell types resided in the myocardium. They were reported to transdifferentiate into cardiomyocyte *in vivo* at acceptable low rates (Toma et al. 2002; Eschenhagen et al. 2017). Although the ultimate cell fate remained controversial and outcomes of the clinical trial were variable, MSCs transplantation was shown to be safe (White et al. 2016; Pandey et al. 2017). The induction of survival factors, angiogenic factors, or growth factors expressed by MSCs might be applied to enhance its therapeutic potency, but the evidence of transdifferentiation of these cells into cardiomyocytes remains scant at best.

Cardiac stem cells (CSCs). Most studies have focused on the cell population expressing C-kit or Sca1 in the heart since they were reported to eventually display cardiomyocyte markers in specific conditions *in vitro* as well as *in vivo* (Zaruba et al. 2010; Jesty et al. 2012). However, several of these research have later been challenged, and the actual therapeutic benefit of these cells is now heavily questioned by the scientific community (Van Berlo et al. 2014; Elhelaly et al. 2019).

Embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs)-derived cardiomyocytes. Noticeable results underline the feasibility of these cells engrafted in the heart of small and large animals, including primates

(Lalit et al. 2014; Shiba et al. 2016; Liu et al. 2018). The efficiency of *ex vivo* differentiation of ESCs or iPSCs to immature cardiomyocytes was reported to exceed 80% (Cao et al. 2013; Burridge et al. 2014). Despite their origin, these cardiomyocytes were able to couple with the host myocardium electromechanical circuit, although arrhythmias were observed in the few treated primates. However, the fact that they are allogeneic needs to be underlined, thus requiring the immunosuppression of recipients and bearing the risk of teratoma formation arising from undifferentiated cells resisting the differentiation process (Nussbaum et al. 2007).

In summary, we are still searching for any effective cell-based therapy for the treatment of cardiovascular diseases. Although the improvements of cardiac function from research findings or small-scale trials in the past decade have elicited great excitement, the actual contribution of cell-based therapy still requires the clarification of the molecular mechanisms involved.

1.1.3.2 Direct reprogramming

The concept of reprogramming one cell type to another has unlocked a fascinating opportunity for regenerative medicine. The discovery from stem cell studies has inspired the idea of rebuilding the heart by reprogramming cardiac fibroblasts, which constitute roughly 60% of all heart cells, into cardiomyocytes and therefore bypassing the risk of tumorigenesis and diminish the fibrosis (Xin et al. 2013; Gourdie et al. 2016). An initial demonstration of this concept was reported by the identification of a GMT cocktail including three cardiac transcription factors GATA4, MEF2c, and TBX5, which was shown to stimulate partial transdifferentiation of cultured murine fibroblast into cardiomyocyte-like cells (Ieda et al. 2010). Later, the GMT cocktail was supplemented by the fourth factor HAND2 (GHMT cocktail) to increase the reprogramming efficacy up to ~20% (Song et al. 2012).

The following studies have focussed on enhancing the reliability, safety, and effectiveness of this process. Efforts were made for stoichiometry factors selection of the cocktail, development of appropriate methods to

deliver factors of interest or searching for specific cardiac cell subsets (Wang et al. 2015b; Miyamoto et al. 2018). Several pathways were manipulated to improve the transdifferentiation ratio. These included TGFβ, AKT1, WNT, and Notch1 signalling (Ifkovits et al. 2014; Zhou et al. 2015; Gourdie et al. 2016). *In vivo* delivery of GMT or GHMT cocktails by retroviruses were shown to reprogram directly cardiac fibroblasts activated after myocardial injury (Qian et al. 2012; Song et al. 2012). Besides, the introduction of several miRNAs also enhanced reprogramming effectiveness *in vivo;* these included miRNA-1, miRNA-133, miRNA-208, and miRNA-409 (Nam et al. 2013; Jayawardena et al. 2015).

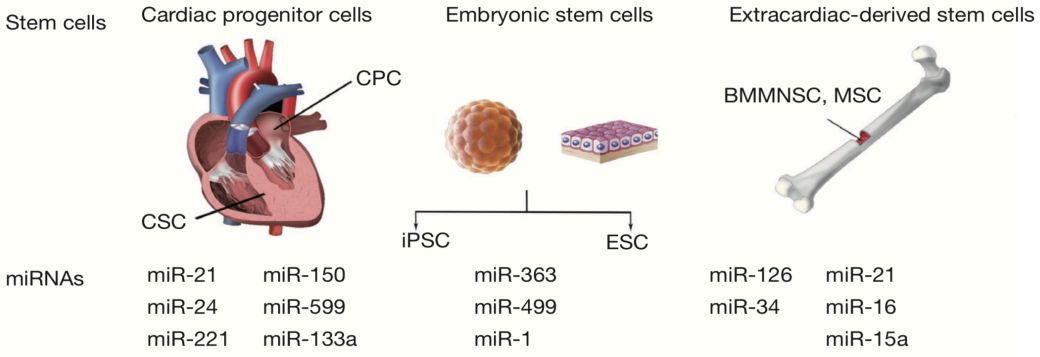

Figure 1.3: Several miRNAs are involved in heart regeneration. miRNAs have been used to promote cardiomyocyte proliferation, enhancing the differentiation and survival of transplanted cells or reducing the adverse effects of non-myocyte cells in the heart (Zhu et al. 2016).

Of note, human fibroblasts are more refractory to transdifferentiation, as the administration of the indicated cocktails required the aids of MESP1, ESRRγ, or myocardin to function (Islas et al. 2012; Fu et al. 2013). A virus-free method, using combinatorial treatments of the pluripotent gene cocktails and nine different chemicals, was reported to efficiently enhance human fibroblast transdifferentiation into cardiomyocytes, which eventually displayed spontaneously beating (Cao et al. 2016). Despite the variability of cardiac reprogramming efficiency, the TGFβ and WNT signalling pathways were confirmed to share similar roles in mouse and human cells, thus underlined their crucial contributions to this process (Liu et al. 2016b; Mohamed et al. 2017). Although the concept of reprogramming seems

exciting, several obstacles remain to be addressed before its application in heart regeneration. We need to clarify epigenetic modifications that occur during the development to set the right stage of transdifferentiated myocytes compared with the pre-existing tissue. At the same time, the longevity of newborn cells requests verification *in vivo.*

1.1.3.3 Stimulation of the innate cardiac regenerative capacity

Cumulative evidence indicates that cardiomyocytes slowly replicate from 0.5% to 2% per year in intact adult human and murine hearts (Laflamme and Murry 2011; Eschenhagen et al. 2017). Therefore, natural renewal is inefficient and incapable of recovering the injured heart but serves as a precious regenerative source. Stimulation of endogenous cardiac myocyte proliferation has been considered as one of the major paths in cardiac regenerative therapy (Singh et al. 2018; Heallen et al. 2019). Hence, multiple factors inducing cardiomyocyte turnover have been delivered to the heart using different modalities, including protein-coding viral or non-viral vectors, small RNAs, and small molecules (Grigorian Shamagian et al. 2019).

Gene therapy. Many attempts were made to understand the molecular mechanisms behind the cardiomyocyte cell division. Various factors, cell cycle regulators, and intrinsic signalling pathways were identified to play the crucial roles in cardiogenesis, cardiomyocyte maturation, and epigenetic modifications. The evolution of vectors and delivery systems has provided researchers powerful weapons to intervene in particular genes or pathways of interest (Wolfram and Donahue 2013). Early efforts were made by Dr. J. Isner and colleagues to inject intramuscular naked plasmid encoding VEGF that could augment collateral development and tissue perfusion (Tsurumi et al. 1996). As a non-integrative approach, plasmid DNA delivery is straightforward and avoidable the risk of tumorigenicity. However, it failed to convince functional improvement was significant through several trials and the transgene expression was simply low (Vale et al. 2000; Mendiz et al. 2011). Afterwards, viral vectors were viewed as an

effective method to deliver target genes to the heart. Lentiviral, adenoviral, and adeno-associated virus-based vectors are the most common viral vectors currently used. Multiple genes were introduced to the heart using viral vectors to stimulate endogenous cardiomyocyte proliferation. The combinatorial delivery of vector expressing cell cycle regulators including CDK1/CCNB/CDK4/CCND increased significantly the rate at which adult cardiomyocytes underwent cell division (Mohamed et al. 2018). Studies in rat and pig models for the overexpression of cyclin A2 mediated by an adenoviral vector protected the infarcted heart and induced cardiac regeneration (Woo et al. 2006; Heallen et al. 2019). Along the same concept, AAV9 expressing Salvador shRNA was injected in the infarcted heart to modulate Hippo pathway activity and improve heart function (Leach et al. 2017). The rise of CRISPR/Cas9 technology provides a powerful tool for the field of genome editing, which has been explored to correct the mutation of some essential genes in the heart. Several attempts were made to deliver CRISPR/Cas9 components to correct *Myh7* and *Mybpc3* mutations in hypertrophic cardiomyopathy (Cannatà et al. 2020). Moreover, CRISPR/Cas9 mediating *Mybpc3* correction was achieved at high yield in human preimplantation embryos (Ma et al. 2017).

Extracellular vesicles. These are a heterogeneous group of cellular particles comprising two major classes: exosomes and microvesicles. The exosome is derived from a multivesicular endosome fused with the cell membrane, while the microvesicle is a highly variable class regarding their origin and secretion process (De Jong et al. 2014; de Abreu et al. 2020). An extracellular vesicle can comprise many forms of RNA (mRNAs, miRNAs, non-coding RNAs), DNA, lipids, or proteins (Valadi et al. 2007; O'Brien et al. 2020). Recent evidence supports their roles in vascular homeostasis, cardiac fibrosis, or cardiac pathological progression (Bang et al. 2014; Beltrami et al. 2017). Exosomes from the cultured human cardiosphere-derived cell (CDC) were shown to promote cardiomyocyte proliferation and inhibit apoptosis. These exosomes were able to preserve the heart function and structure after 42 days from myocardial infarction by upregulating

miR-146a expression (Gallet et al. 2016). In another study on cardiac progenitor cells (CPCs), extracellular vesicles derived from CPCs released several miRNAs such as miR-210, miR-132, and miR-146a-3p, to suppress apoptosis in cultured cardiac cells and maintain the cardiac function of infarcted mice (Barile et al. 2014). Interestingly, current reports suggest that extracellular vesicles can deliver full mitochondrial genomes to recipient cells, subsequently recovering their respiratory activity and enhancing cell survival (Sansone et al. 2017; Puhm et al. 2019).

MicroRNAs (miRNAs) and Long non-coding RNAs (lncRNAs). miRNAs have been studied extensively since their discovery in the early 1990s by Victor Ambros and colleagues (Lee et al. 1993). MiRNAs are described as small regulatory RNAs that modulate gene expression at the post-transcriptional level by pairing with complement mRNA at target sequences (Thum et al. 2007; Small and Olson 2011; Hashimoto et al. 2018). miRNAs are transcribed and processed from specific genes located within introns or exons of coding genes (Ha and Kim 2014); transcription starts from the hosting promoter or from self-promoters (MacFarlane and R Murphy 2010). Expression of miRNAs can be regulated by miRNA gene modification, RNA editing or methylation (Misiewicz-Krzeminska et al. 2019). The most common function of miRNAs is to reduce gene expression, by which sufficient matching to target mRNAs leads to their degradation while partial matching mostly inhibits their translation (Bartel 2004). miRNAs regulate many biological processes, including cardiogenesis and development of cardiovascular disease. One of the early reports about miRNAs controlling cardiomyocyte proliferation was the identification of the miR-15 family, which regulates postnatal mitotic arrest (Porrello et al. 2011). Furthermore, our group has provided striking evidence that miR-199a-3p and miR-590-3p are able to induce proliferation of postnatal and adult cardiomyocytes (Eulalio et al. 2012). Advances in delivery methods showed that the administration of a miRNA-302 hydrogel improved local cardiomyocyte proliferation and increased the number of myocytes in adult mouse heart (Wang et al. 2017). The crosstalk between ubiquitination and

miRNAs we reported recently, when infarcted CD57 mice injected with miRNA-1 that the function of the proteasome was associated with miRNA expression (Wei et al. 2019). Although most therapeutic miRNAs for cardiac regeneration are still in preclinical developing stages, a few miRNAs have reached clinical trials for other applications (Janssen et al. 2013; Beg et al. 2017). In particular, MRG110 (a synthetic miRNA-92 inhibitor) is one of the few ongoing trials for heart failure treatment (Hanna et al. 2019).

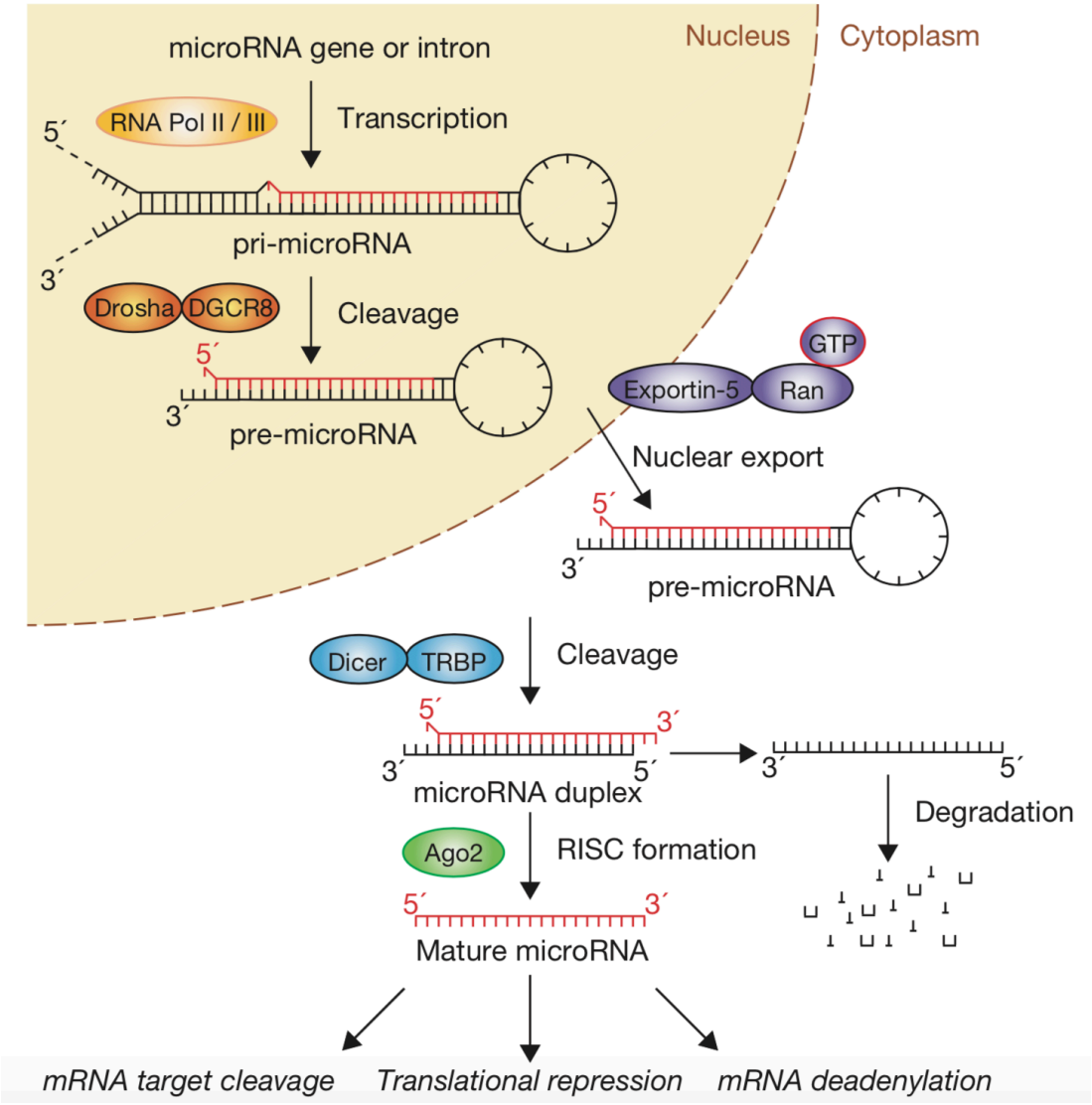

Figure 1.4: miRNA maturation process. The pri-miRNA is transcribed from miRNA gene or intron and then cleaved by the Drosha-DGCR8 complex in the nucleus to create the pre-miRNA. After expor to the cytoplasm, pre-miRNA is processed further by Dicer-TRBP to form miRNA duplex. Ago2 loads miRNA duplex into RISC, which guides the functional miRNA to its target mRNA (Winter et al. 2009)

Besides miRNAs, the transcriptional activity of the mammalian genome mainly results in the generation of non-coding RNAs (Kashi et al. 2016). In particular, long non-coding RNAs (lncRNAs) are a heterogeneous class of RNA transcripts of over 200 nucleotides comprising sense, antisense, intronic, bidirectional, intergenic and enhancer subclasses (Abbas et al. 2020; Tang et al. 2020). Over the last few years, mounting evidence have indicated that lncRNAs interact with DNA, mRNA, and even miRNAs to controlthe splicing, transcriptional or post-translational modification of the factors involved in various cellular function (Kuo et al. 2019; Kazimierczyk et al. 2020). LncRNAs are connected with ubiquitination to regulate the proteolysis, as in the case of lncRNA-CCDST promoting binding of E3 ligase MDM2 with the substrate DHX9 in cervical cancer (Ding et al. 2019). As far as cardiomyocyte proliferation is concerned, this process is also under the control of different lncRNAs. For example, the upregulation of lncRNA ECRAR promoted cytokinesis of postnatal and adult cardiac myocytes by activating the ERK1/2 pathway (Chen et al. 2019). The *Sirt1* antisense lncRNA overexpression *in vitro* was reported to induce mitosis while improving adult mice survival and heart function after infarction (Li et al. 2018).

Small molecules: It is estimated that roughly 40% of the cell volume is occupied by large biopolymers such as DNA, RNA, proteins, and other macromolecules. Besides, the cells also contain small molecules, which are a heterogeneous group of low molecular weight molecules such as lipids, sugars, drugs, and other xenobiotics (Gerry and Schreiber 2020). This group of molecules can interfere with multiple biological processes, including cell signalling, membrane trafficking, DNA replication, and epigenetic modifications (Cañeque et al. 2018). In terms of administration, small molecules are preferably compared to nucleic acids or proteins to treat cardiac disease since they can be delivered orally and uptaken directly by the body (Petrone and DeFrancesco 2018). A major issue is identifying suitable small molecules for cardiac regeneration, which is a challenging proposition given by the complexity of the biological pathways

regulating endogenous cardiomyocyte proliferation. A few small molecules have been described to enhance cardiomyocyte proliferation and hold a promising potential for cardiac regeneration. For example, from a large scale screening of around ~147.000 small molecules targeting the *Nkx2.5* gene, Schneider and colleagues reported the discovery of sulphonyl-hydrazone (Shz), which was able to induce cardiac cell fate of adult progenitor cells (Sadek et al. 2008). More recently, a novel compound (TT-10) derived from the fluorination of TAZ-12 was reported to increase cardiomyocyte proliferation *in vitro* as well as decrease cardiac remodelling in adult mice after MI through its anti-apoptotic effects (Hara et al. 2018).

1.1.4 The complexity of cardiac regeneration

Regeneration is commonly defined as the process by which lost or damaged tissue is replaced by new tissue. Although most of the works so far conducted has aimed at the creation of new cardiomyocytes since they are the main functional population in the heart, we have to remember that the myocardium also comprises other cell types, which collectively account for more than 60% of the cells in an adult heart. Thus, the induction of cardiomyocyte proliferation is crucial but merely not enough to reconstitute the injured tissue. In broader terms, trauma can be found at different levels of the heart: cellular death, tissue architecture disruption, lack of proper vascularisation, impaired interaction with white blood cells (Grigorian Shamagian et al. 2019). Consequently, an ideal scenario is to rebuild the heart based on the orchestrations of multiple actions: remuscularization, electromechanical coupling, and the modulation of inflammatory and immune response (Bertero and Murry 2018; Cao and Poss 2018).

Remuscularization. This term refers to the myocardium restoration with new cardiomyocytes and subsequent blood vessel formation and connective tissue. In the past, non-myocyte resident cells were viewed as spectators during the regenerative process. However, it has become clear that these cells form a scaffold to support the newborn myocytes organisation and promote their survival (Gray et al. 2018). Moreover, recent

information concludes that regenerative programs are set as different gene expression profiles in all the cardiac cell types (Goldman and Poss 2020). Therefore, a single intervention on cardiomyocytes for a complex organ like the heart is not likely to be successful (Bertero and Murry 2018).

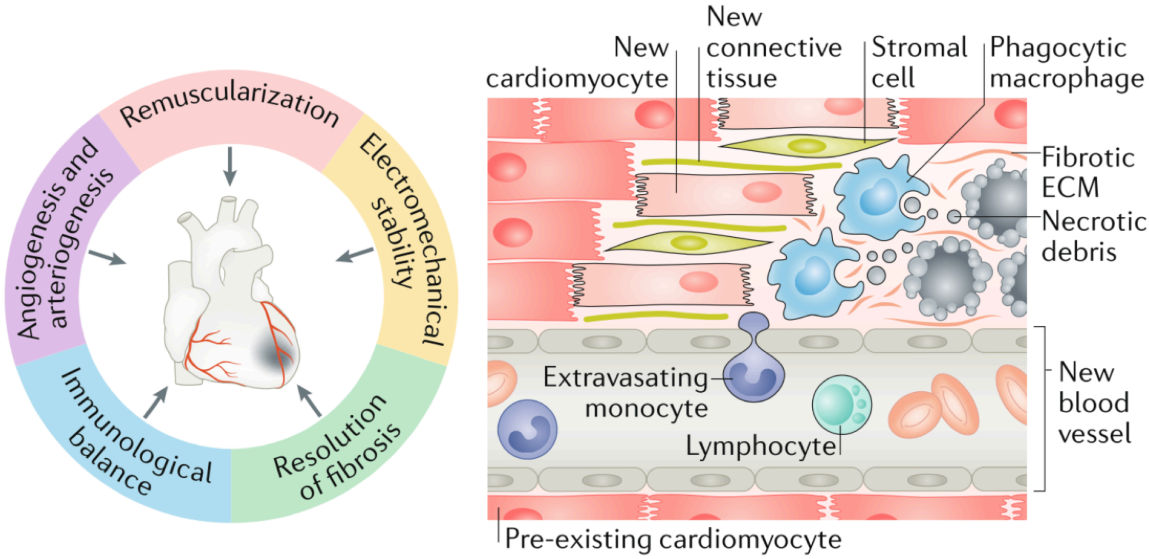

Figure 1.5: Heart regeneration properties (Bertero and Murry 2018). Sufficient cardiac regeneration requires the regeneration of myocardium by new myocyte, blood vessel, and connective tissue that needs to couple with electromechanical circuit of existing tissue. Immune response also modulates this process.

Electromechanical coupling. As we have learned from development lessons, reassembly of the heart requires the generation of fully functional cardiomyocytes in terms of excitation-contraction machinery composition. Aside from finding new sources of cardiomyocyte, tissue synchronisation has always been one of the most troubling issues for cardiac regenerative medicine (Heallen et al. 2019; Sadek and Olson 2020). In cytological terms, this means heart regeneration can only achieve from accomplishing all aspects of reconstruction of the transverse tubules, gap junctions, and ion channels, besides forming new myofibrils within the newly generated cells.

Modulation of the inflammatory and immune responses. An adequate tissue replacement after a myocardial injury is regulated by immune and inflammatory cells. In particular, an appropriate inflammatory response is crucial to eliminate necrotic cells, trigger angiogenesis, and fibroblast

ingrowth (Liehn et al. 2011; Prabhu and Frangogiannis 2016). However, the blunting of this inflammatory response is crucial to start the recovery phase. Due to indistinct boundaries between beneficial and harmful contributions, inflammatory and immune cells are somehow underrated on the heart regeneration path. Excessive reactions may lead to the collateral damage of reforming tissue and slow down the healing (Sattler et al. 2017). In this respect, a thorough understanding of the actual molecules secreted by white cells present in the blood or resident in the myocardium might lead to the definition of therapeutic proteins that might improve new cardiomyocyte and blood vessel formation to enhance the functional outcome of regenerative approaches.

In conclusion, heart regeneration is a daunting task that requires more in depth investigation to set up the right stage for multiple cell populations and cellular factors building up the functional tissue.

1.2 The ubiquitination machinery

In the early days of protein metabolism studies, proteins were defined as an everlasting molecule that, once produced, stayed indefinitely with the host (Ohsumi 2014; McDowell and Philpott 2016). Later on, this dogma was challenged by the observation of proteolysis *in vivo*. In this experiment, the animals were fed with the foods containing labeled atoms and the signal was detected in their tissue that indicating some form of protein turnover (Bloch and Schoenheimer 1939; Ohsumi 2014). In the 1980s, a milestone of ubiquitination study was introduced by Hershko, Ciechanover, Rose, Varshavsky with other colleagues for the discovery of this system (Ciechanover et al. 1980; Hershko et al. 1980).

Ubiquitylation is a reversible post-translational modification in which ubiquitin(s) are covalently linked with recipient proteins at lysine residue through an enzymatic cascade (Callis 2014; McDowell and Philpott 2016). This modification occurs in virtually all cellular activities, including cell cycle progression, apoptosis, intracellular trafficking, and viral infection (Swatek

and Komander 2016). In particular, most intracellular proteins are degraded through the ubiquitin-proteasome system, whereas extracellular proteins are taken up by endocytosis and degraded via lysosomes (Lecker et al. 2006). Tightly control of protein turnover is crucial for maintaining the proper function of mammalian cells. For example, rapid modifications of specific proteins allow host cell adaptation to physiological shifts. This is the case of UBI4, an inducible factor of stress response to DNA damage or heat shock, which provokes the rapid formation of ubiquitin molecule (Gemayel et al. 2017), or of the E3 ligase PIRE, which modulates the ROS production to defend from a bacterial infection (Lee et al. 2020).

The ubiquitination system includes diverse types of factors. In particular, there are 2 members of ubiquitin activating enzyme (E1), approximately 40 members of ubiquitin conjugating enzyme (E2), and over 600 ubiquitin ligases (E3) in mammalian cells (Clague et al. 2015). Besides, the genome also encodes around 100 deubiquitinating enzymes and accessory chain elongation factors called E4 (Koegl et al. 1999; Nielsen and MacGurn 2020).

1.2.1 General mechanism

Since the ubiquitination was first identified as a proteolytic pathway, it was often associated with the proteasome to be collectively named the ubiquitin-proteasome system. However, this nomenclature partially explains its function since countless research of ubiquitylation driving non-degraded modifications of the substrates published to date testify.

The ubiquitination process starts when ubiquitin is activated by a covalent linkage between its C-terminus (glycine 76) and a cysteine residue of E1, then activated ubiquitin is transferred to a cysteine residue of E2 before the E3 mediates its attachment canonically to a lysine residue of a substrate protein (Pagan et al. 2013). Depending on the distinct chain topology given by the ubiquitination, substrate proteins can have various outcomes. For example, the mono-ubiquitylation can alter protein-protein interactions, homotypic chain linkage at lysine 11 or 48 primes the receiver protein to proteolysis while heterotypic chain addition onto lysine 63 can

mediate the endocytosis or protein assembly (Clague et al. 2015; McDowell and Philpott 2016). Therefore, the prediction of protein behavior followed ubiquitylation requires an intensive analysis of chain formation and its localisation on the substrate.

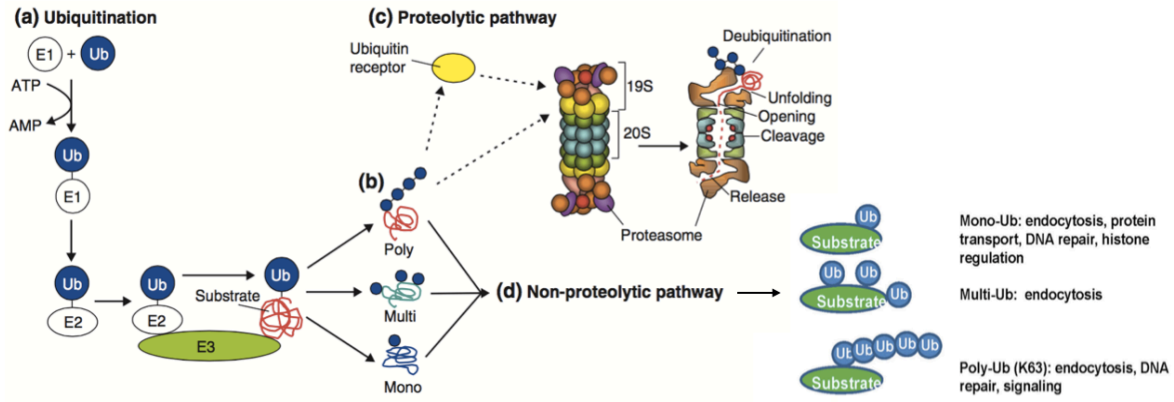

Figure 1.6: Ubiquitination and its roles in various cellular processes adapted and modified from (Kaiser and Huang 2005). (a) Ubiquitination comprises several enzyme types and catalyses ATP-dependent reactions. (b) Polyubiquitylation of substrate results in proteolysis. (c) The proteasome degrades ubiquitylated substrates and deubiquitinase reuses ubiquitin molecules. (d) Different types of modifications drive various substrate outcomes.

1.2.2 Components

1.2.2.1 Ubiquitin

Staying at the heart of the ubiquitylation process is the tiny, 76 amino acid-protein ubiquitin, which is highly conserved and expressed universally. Across various eukaryotic kingdoms, ubiquitin has changed only a few residues between plants, fungi, and animals (Zuin et al. 2014). The interchangeable function of ubiquitin was examined by providing ubiquitin derived from yeast to plants, and the subsequent results presented no significant differences was found in the plant phenotype (Ling et al. 2000). This experiment revealed among those that indicated that the ubiquitous function of ubiquitin had been maintained along with evolution.

Ubiquitin molecule has a globular structure composed of five β-sheets encompassing an α-helix packaged tightly to enhance protein stability and

prevent heat denaturation. The C-terminus of ubiquitin is less preserved to interact with multiple target proteins (Dikic et al. 2009). In humans, ubiquitin protein is encoded by four genes. The *UbB* and *UbC* genes encode polyubiquitin chain precursors consisting of nine ubiquitin molecules and associate with the proteasome for substrate proteolysis. While the *UBA52* and *UBA80* genes encode ribosomal subunits comprising a single ubiquitin at its C-terminus (Clague et al. 2015; Tramutola et al. 2016).

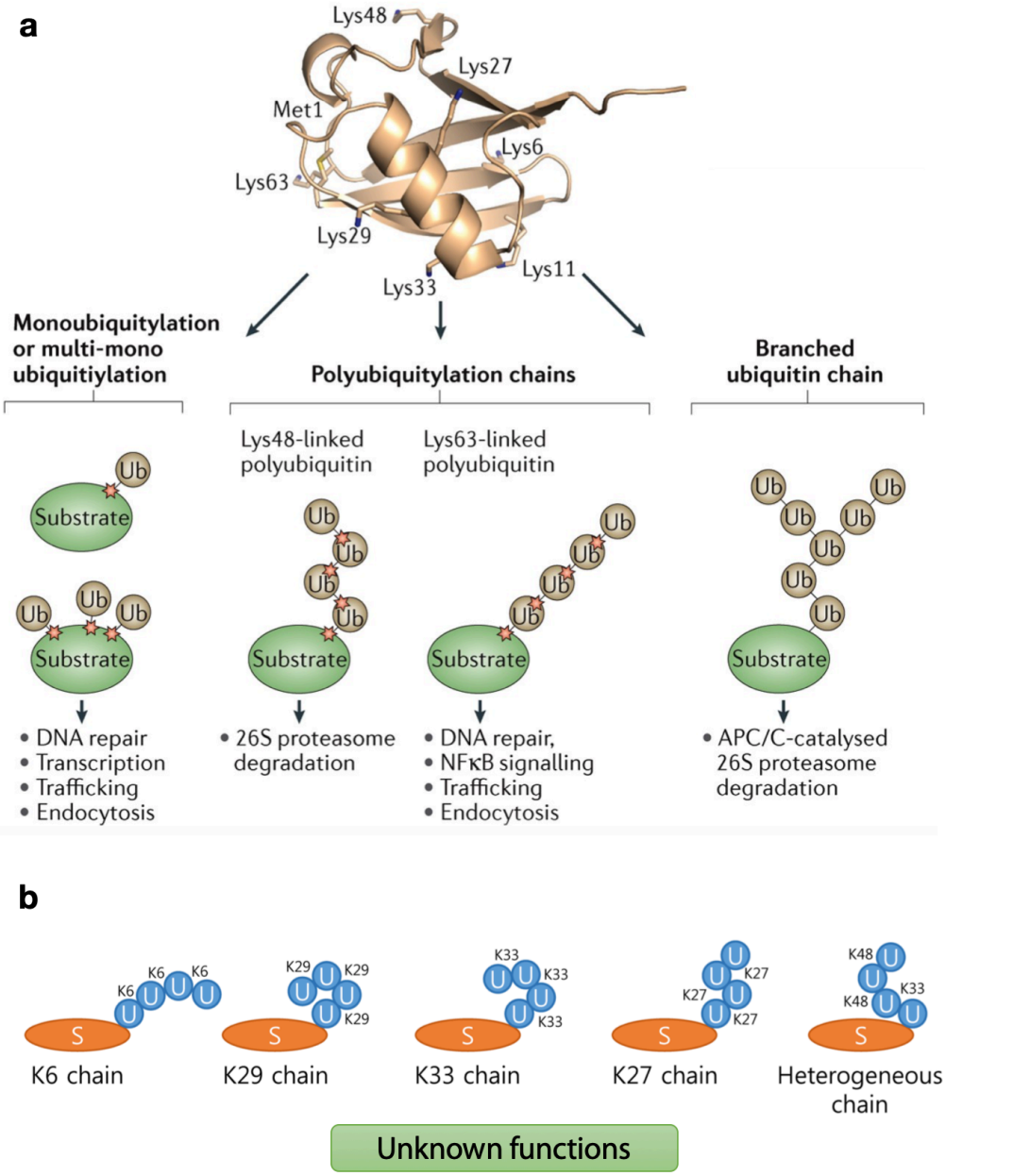

Figure 1.7: Various ubiquitin chain linkages and their functions adapted and modified from (Park and Ryu 2014; Buetow and Huang 2016). **a.** Types of ubiquitylated modifications with known functions. **b.** Ubiquitylation at the residues

with unknown function.

As mentioned before, ubiquitin chains can be conjugated to each other through eight internal residues in order to form a variety of chain linkages. The residues involved are methionine 1 at the N-terminus and seven lysine residues at positions 6, 11, 27, 29, 33, 48, and 63 (Dikic et al. 2009; Winget and Mayor 2010). Intriguingly, a significant number of ubiquitin molecules are detected as monoubiquitin conjugates in mammalian cells. For example, in HEK293 cells, approximately 60% of total ubiquitin chains are in the form of monomeric protein (Kaiser et al. 2011).

Multiple pathways and factors were found to be regulated by ubiquitin-like proteins in a similar manner (Hoeller et al. 2006). Ubiquitin-like proteins share an α-helix and a β-sheet structure analogous to ubiquitin, but they principally mediate the interactions of proteins with other macromolecules. Moreover, their structures also recall some traits of prokaryotic proteins (Hochstrasser 2009). The ubiquitin-like protein modifications include the sumoylation (involving the addition of the SUMO polypeptide), neddylation (NEDD8), ISGylation (ISG15), and fatylation (FAT10). Additional ubiquitin-like proteins are ATG8, ATG12, URM1, and UFM1 (Hoeller et al. 2006; Cappadocia and Lima 2017).

1.2.2.2 Ubiquitin activating enzyme (E1)

The ubiquitylation process is initiated by the ubiquitin activating enzyme (E1). In brief, the activation begins when the C-terminus of ubiquitin is adenylate by E1 using energy from ATP hydrolysis. This reaction generates high energy ubiquitin-AMP self-transferred onto a cysteine residue of E1 (Callis 2014; McDowell and Philpott 2016). After this step, a thioester bond is formed between E1 and the C-terminus of “activated” ubiquitin. This binding results in a conformational change exposing a binding site to ubiquitin conjugating enzyme (E2) and release AMP (Rape 2018). Generally, E1 comprises four domains: a binding domain consisting of two motifs to turn “on” and “off” adenylation, a catalytic domain containing the active cysteine, an intermediate layer of four helices assisting the binding of E1

and ubiquitin, and a C-terminus portion interacting with specific E2s (Lee and Schindelin 2008).

There are two E1s in vertebrates to initiate the process of Ubiquitylation. Of these, for several years, UBE1 was believed to be the sole E1. The discovery of UBA6 has then added a new layer of complexity (Jin et al. 2007). UBE1 and UBA6 are unrelated factors, as the overlapping of their structures is only approximately 40% (Schulman and Harper 2009). Even though both proteins are expressed in various cell types, the UBE1 level is far more abundant than that of UBA6. In terms of activity, UBA6 is responsible for less than 1% of total ubiquitylation activity, which also explains why its existence has escaped the identification for many years (Hyer et al. 2018). UBE6 is known to interact uniquely with the USE1 E2 enzyme.

1.2.2.3 Ubiquitin conjugating enzyme (E2)

In the subsequent step of ubiquitylation, the ubiquitin conjugating enzyme (E2) continues the cascade by first accepting “activated” ubiquitin at a cysteine active site and dissociating from E1. Then, it interacts with cognate E3 ligase(s) to recruit the substrate (Ye and Rape 2009). There are two main types of reactions carried out by E2s: transthiolation (movement of a thiol group between two molecules) and aminolysis (movement of a thiol group to an amino group) (Stewart et al. 2016). Approximately 40 E2 members have been identified in humans. Most of them are small proteins, except UBE2O3, the mass of which exceeds 230 kDa. Due to their relatively small size, E2s bare a single-core domain for catalysis named UBC comprising 140-200 amino acids that is formed by four α-helices and one β-sheet (Polge et al. 2015). E2s are classified into four classes according to their structures: class I possesses only the core domain, class II and III require either N-terminus or C-terminus for catalyzing, while class IV contains both of them (Polge et al. 2015; McDowell and Philpott 2016).

A single E2 can interact with one or multiple E3s, and those interactions

can have either weak or moderate strength. Thus, a traditional approach based on pulldown assays is not suitable to identify the physiological paring of E2-E3 in most cases. A low affinity of the E2-E3 interaction may be an advantage for ubiquitin chain formation. As E2 cannot be "used" by E1 while E3 is binding, the release of E3 is needed for initialising the sequential reaction. The interchange between attachment and detachment usually takes time and slows down the turnover of E2. In some conditions, accelerating chain formation is achieved through auxiliary site interaction of E2-E3 outside the canonical domain (Eletr et al. 2005; Ye and Rape 2009). Remarkably, ubiquitin chain topology can be determined alone by E2, as several of these proteins are able to synthesize K48 polyubiquitination without E3 (Choi et al. 2015; Polge et al. 2015).

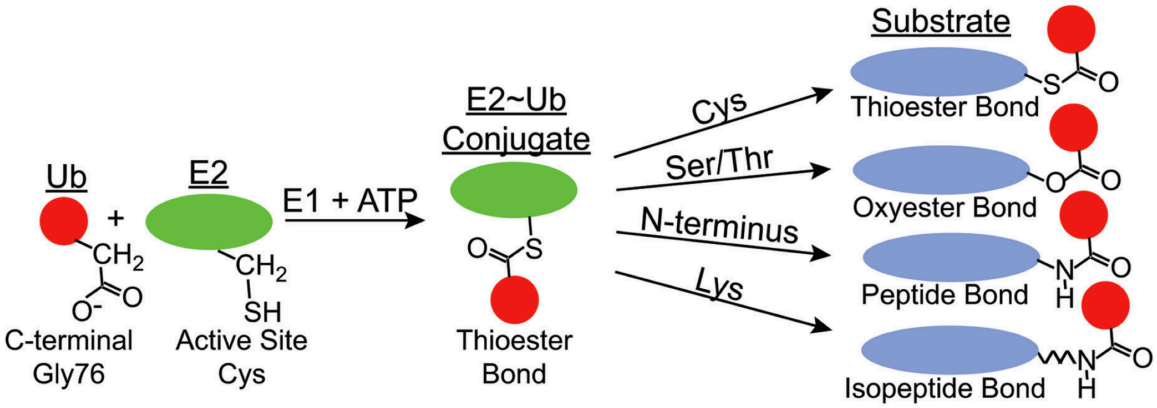

Figure 1.8: Different ubiquitin linkages based on types of interaction between E2 and substrates (Stewart et al. 2016). E2 conjugating enzyme transfers ubiquitin onto different residues of the substrates.

In the cells, E2s can play different roles since several of these proteins participate in the initiation of the ubiquitin chain, including the UBE2D family, while others are more prone to the chain elongation. In the case of UEV, it has several variants, which contains the UBC domain but lacks a cysteine active site. Instead of directly performing the ubiquitylation, UEVs bind other active E2s and act as cofactors in the catalysis (Pickart and Eddins 2004). Although the conjugation of activated ubiquitin to an E2 is the most common activity in the cells, these proteins also possess a low ubiquitin transfer rate in undefined mechanisms. Additionally, some of the

E2s have highly equivalent isoforms, for example UBE2A is 96% similar to UBE2B (Polge et al. 2015). Therefore, it remains still unclear how such a relatively small number of E2s can work in conjunction with a large number of E3s in a highly specific manner and whether redundancy exists.

1.2.2.4 Ubiquitin ligase (E3)

Ubiquitin ligase (E3) is responsible for the transfer of “activated” ubiquitin to substrates. In detail, these factors mediate specifically the recognition of target proteins. E3s are the largest family of enzymes in the ubiquitylation machinery, with an estimate that the human genome encodes more than 600 of them (Senft et al. 2018). Generally, all E3s harbour an interacting domain with E2, although the structures and their modes of action seem to vary. Interestingly, multiple E3s with various structures may facilitate similar interactions (McDowell and Philpott 2016). So far, E3s have been classified into three major subgroups according to their characteristic domain as well as the ubiquitin transfer mechanism (Morreale and Walden 2016).

RING E3s (Really Interesting New Gene). They are the most abundant family since approximately 600 of RING E3s were found in humans (Morreale and Walden 2016). A canonical RING domain consists of 30-100 residues motif coordinated by two zinc ions; the structure of around 340 RING E3s has been validated. U-box enzyme is a sub-group of RING E3 proteins that possess a classical domain lacking the zinc coordination part, and eight of them have been detected in human cells. Both classical RING and U-box E3s catalyse the ubiquitin transfer directly from an E2 to the substrate.

RING members can catalyse the reaction as monomers, homodimers (the complex of two molecules of a RING ligase), or heterodimers (the complex of two different RING ligases). In some particular circumstances, they form complexes of multisubunit E3s, such as Cullin-RING ligases or APC/C complex. With more than 300 substrate receptors confirmed in humans, the Cullin family is responsible for 20% protein degradation carried out by proteasome (Clague et al. 2015). Furthermore, RING E3s can

be regulated through neddylation and phosphorylation.

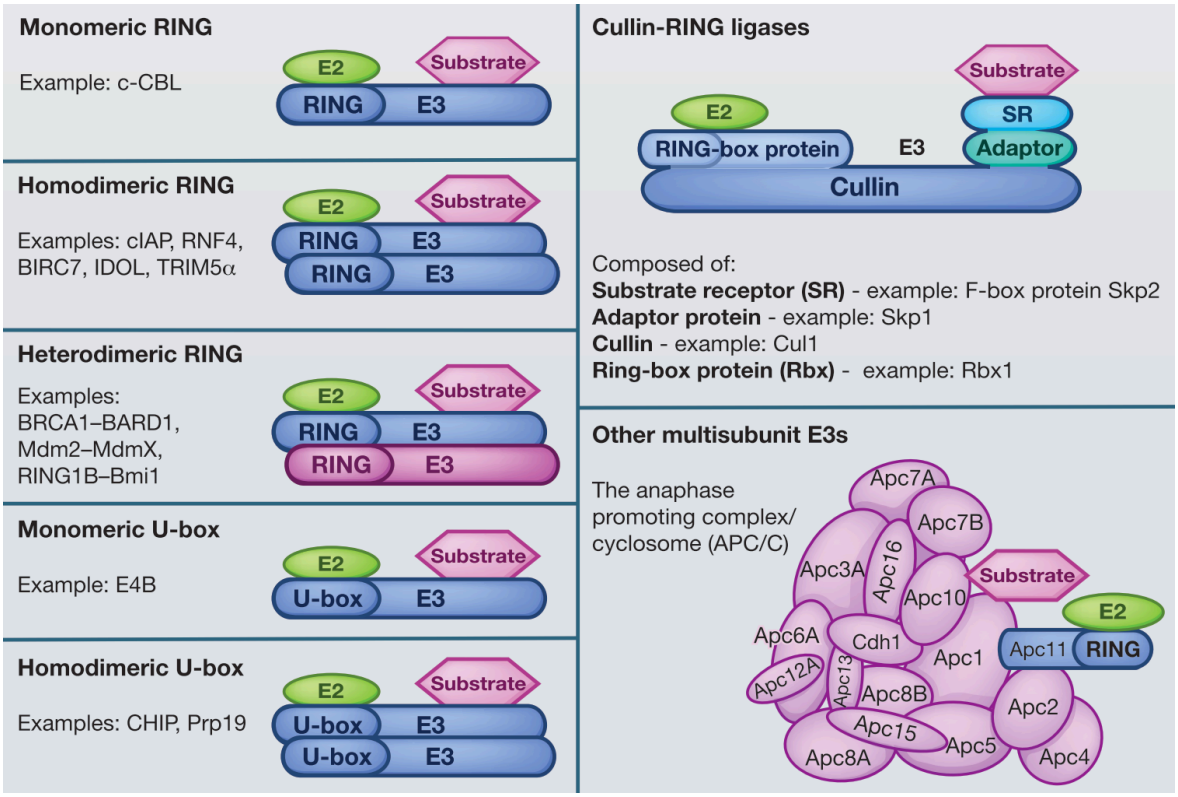

Figure 1.9: Numerous types of RING E3 ligases (Morreale and Walden 2016). These can be recognised as classical RINGs, U-box, Cullin-RING or multi-subunit ligases.

HECT E3s (Homology to E6AP C-Terminus). These were named after the discovery of their prototypical domain within the E6-AP protein of human cells. Unlike the previous E3s group, HECT E3s perform ubiquitin transfer through a two-step reaction: the HECT domain facilitates "activated" ubiquitin acceptance from E2, and the binding domain at the N-terminus recruits the substrate (McDowell and Philpott 2016). The HECT domain comprises 350 amino acids and locates at the C-terminus (Callis 2014; Buetow and Huang 2016). In order to interact with E2, two lobes of HECT domain work collectively to form a flexible hinge in which the N-terminal lobe connects with E2 at one side, and the C-terminal lobe at the other side contains an active cysteine to catalyse the transfer (Morreale and Walden 2016). Substrate specificity is regulated by N-terminal extensions, by which these proteins can be divided into three subfamilies. These include NEDD4,

HERC, and leftover members. So far, 28 HECT E3s have been identified in humans.

RBR E3s (RING-IBR-RING). They are an interesting group of E3 which have been discovered recently. Using a hybrid mechanism, RBR E3s possess RING domains and implement ubiquitin transfer via a two-step reaction. Together, RING1 interacts with E2 and places “activated” ubiquitin onto RING2 to proceed with the conjugation of ubiquitin and the substrate (McDowell and Philpott 2016). Although sharing the core of catalysis, each member of RBR E3s owns an additional domain that is structurally different and involves in intramolecular interactions (Callis 2014). Some of these domains were reported to arrest the enzyme at an auto-inhibited state that allows the rapid catalysis in response to phosphorylation or protein-protein interaction (Morreale and Walden 2016). 14 members of RBR E3s have been identified in humans.

Of note, HECT E3s determine the substrate specificity as they receive “activated” ubiquitin before transferring it to the substrate, whereas in RING E3s catalytic reaction, E2 plays a determining role (Stewart et al. 2016).

1.2.2.5 Ubiquitin accessory chain elongation factor (E4)

As mentioned before, polyubiquitin chain linkages are required by ubiquitination to drive the degradation of substrates. In a classical manner, single ubiquitin moieties are added sequentially to the chain, and this cycle is repeated until the completion. Nonetheless, the question of how to mediate concurrently the formation of polyubiquitin and branched ubiquitin chain is still poorly understood. Relevant to this problem, Manfred and colleagues initially identified an accessory elongation factor, called E4. In the absence of E4, the formation of the ubiquitin chain was insufficient for proteolysis *in vivo*. E4 was described to promote the pre-assembly of polyubiquitin chains, which could be conjugated rapidly to a substrate, thus increasing assembly speed (Koegl et al. 1999). After this original report, several E4s have been detected in mammalian cells. Bypassing the classical cascade, the participation of E4 is an obvious benefit for saving

time and energy of proteolysis.

1.2.2.6 Deubiquitinating enzyme (DUB)

Besides controlling cellular protein turnover, ubiquitylation also plays a master role in the governance of signalling pathways. Similar to other post-translational modifications, ubiquitylation is reversible and tightly supervised by deubiquitinating enzymes (DUBs). DUBs can be attracted to particular protein targets based on interacting domains or recognised by specific linkage architectures (Clague et al. 2019). The mammalian genome encodes around 100 DUBs associating with ubiquitin precursor cleavage and recycling of the ubiquitin molecule after the degradation (McDowell and Philpott 2016). Recent data also indicate the contribution of these enzymes to ubiquitin chain editing (Mennerich et al. 2019). Of note, the effects of DUBs on ubiquitin removal correlate with substrate stability as well as function.

Depending on sequence and structure, DUBs are classified into seven subgroups (Reyes-Turcu et al. 2009; Clague et al. 2019): USP, UCH, OTU, MJD, MINDY, ZUP1, and JAMM. Except for JAMM family members, which are zinc-dependent metalloproteases, the remaining DUBs are cysteine-dependent proteases (Clague et al. 2015). Among them, USP is the largest and the most diverse group. Most DUB functions are enigmatic, as these proteins contain various structural motifs for catalysis, ubiquitin binding and protein interaction domains. Hence, it comes to no surprise that they are highly regulated by phosphorylation, ubiquitylation, or sumoylation that affects both their activities and localisation (Reyes-Turcu et al. 2009).

1.2.3 Current approaches for studying ubiquitination

Since the discovery of protein modification by the ubiquitylation process, the identifications of specific substrates and types of modification have always been the holy grails in this field. Several challenges remain to be overcome in this respect, including weak interactions of ubiquitination compartment and substrate, the diversity of factors with redundant

functions, rapid destruction of target protein, and highly dynamic modification in contextual dependent. Many biochemical and bioinformatic tools were either invented or improved to navigate the complexity of this system.

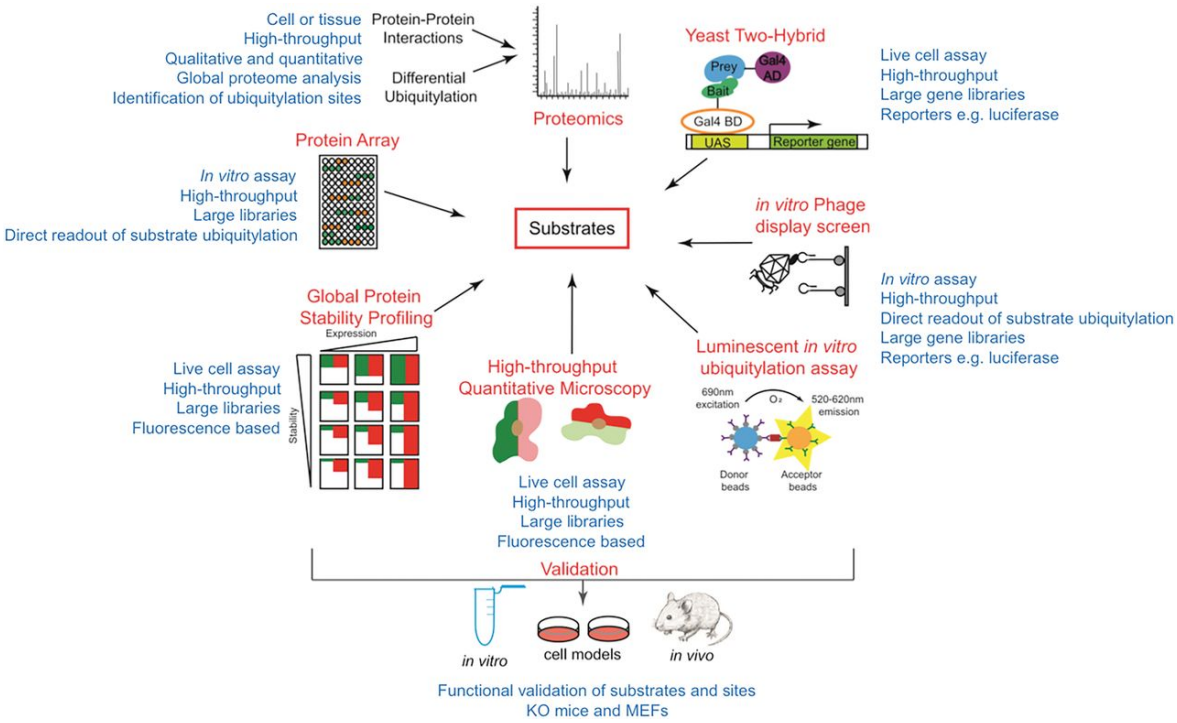

Figure 1.10: Current approaches to identify E3 ligase substrates (Iconomou and Saunders 2016). The identification of substrate can be achieved through these approaches: yeast two hybrid, *in vitro* phage display screening, luminescent *in vitro* ubiquitylation assay, high-throughput quantitative microscopy, global protein stability profiling or protein array.

Biochemical tools. These are the methods of choice when someone aims at investigating the ubiquitination. Overall, strategies encompass the detection of crucial reactivity sites of E3 ligases including the inhibition of DUB releasing ubiquitin molecule from the substrate, the modification of binding and transferring mechanism, and the development of novel adaptor or tracker proteins (Iconomou and Saunders 2016). For example, the new tandem ubiquitin binding entities (TUBEs) can be fused with many ubiquitin binding domains to create a “molecular trap” by which can be pulldown and analysed by mass spectrometry (Hjerpe et al. 2009; Azkargorta et al. 2016). A genetic approach, such as ubiquitin activated interaction traps

(UBAITs) was also developed recently (Maculins et al. 2016). In addition, the orthogonal ubiquitin transfer (OUT) technology has been exploited to profile the targets of UBA1 and UBA6 in mammalian cells (Liu et al. 2017). Remarkably, the latest imaging advances provide excellent tools to visualise and track ubiquitin-related processes in living cells, such as the fragment of Kusabira Green fluorescent protein or GFP-based UPS reporter (GFPu) (van Wijk et al. 2019).

Bioinformatic tools. Several databases have been developed to supply comprehensive resources for ubiquitination research. There are Ubibrowser, UUCD, Ubiprot, mUbiSiDa for ubiquitylated proteins in mammals, or hUbiquitome, which contains experimentally verified ubiquitylation data in human cells.

1.3 Targeting ubiquitination for treatment therapy

Ubiquitination is an important therapeutic target for various human diseases, including cancer, diabetes, and cardiovascular diseases. Drug discovery of this area has achieved significant progress in the last decade since bortezomib was the first proteasome inhibitor approved by the FDA to treat hematologic malignancies in 2003 after a 7 years of clinical trial (Cohen and Tcherpakov 2010). Subsequently, other inhibitors were also licensed for treating lymphoma, lung cancer, or pancreatic cancer. The potential use of such inhibitors in therapy is relatively high, given the numerous linkages between ubiquitination compartments and cellular functions. Unfortunately, proteasome inhibitor is an only group of molecules associating with the ubiquitination used in clinical treatment (Deng et al. 2020).

Several E1 inhibitors were developed but only one entered a clinical trial due to the issue of specificity (Kleiger and Mayor 2014; Huang and Dixit 2016). The development of E2s/E3s inhibitors has lagged although these proteins control substrate specificity and modification types. In 2011, the first inhibitor of an E2 enzyme (CC0651) was found by targeting human E2

UBE2R1 (Ceccarelli et al. 2011; Harper and King 2011). Later, two inhibitors of UBE2N were also discovered to block the transfer of ubiquitin to substrates. Targeting E3 was rewarded with some success and led to the identification of different inhibitors of the E3 ligase MDM2 including MI-63, Mel 23 or idasanutlin, which is currently in phase I clinical trial for blood cancer patients (Yang et al. 2005; Ding et al. 2006; Mascarenhas et al. 2019). A group of E3 ligases comprising the Inhibitor of Apoptosis Proteins (IAP) is the major target of cancer treatment, as these proteins prevent apoptosis and thus allow cancer cell survival. Antagonists of IAP ligase are ongoing clinical trials, such as LCL-161, Birinapant, or AT-406 (Scott et al. 2016).

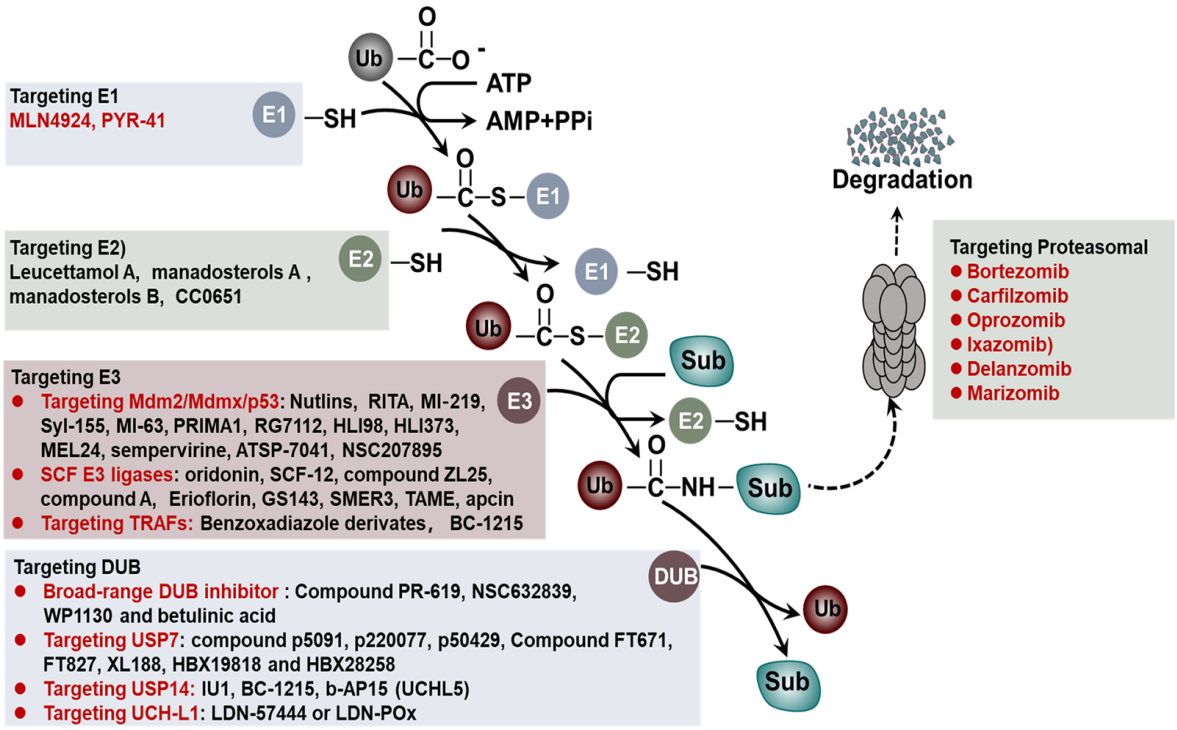

Figure 1.11: Preclinical and clinical trials target ubiquitination components (Deng et al. 2020). Many compounds and factors have been investigated to target ubiquitination at all levels of the reactive cascade.

Interestingly, while much of the work is aimed at blocking the active site of target proteins, ubiquitylation can be exploited in other directions as well. A new set of small molecules call proteolysis targeting chimeras (PROTACs) was constructed to target particular substrates and thus accelerate their proteolysis *in vivo* (Bondeson et al. 2015). Traditional inhibitors or activators

work through interaction with selective pockets to alter the inner structure of target proteins, which leads to unwanted functions in some cases. Therefore, PROTACs have considerable advantages in eliminating abnormal protein synthesis as usually seen in multiple cancers. This new class of degraders has hit clinical trials in 2019 (Mullard 2019).

In the cardiovascular disease scenario, ubiquitination has been studied for years and there is no doubt it plays pivotal roles in all heart functions (Pagan et al. 2013). Dysfunction of the machinery was detected in many heart diseases, which correlates with apoptotic and hypertrophic factor accumulation, alteration of cell mass and disruption of sarcomeric structure (Hedhli and Depre 2009; Henning and Brundel 2017). For example, muscle atrophy F-box (MAFbx) is an E3 that controls the level of calcineurin and its subsequent pathway - nuclear factor of activated T cells (NFAT) -, which controls the maladaptive remodelling of cardiac tissue (Baskin et al. 2014). Among several other evidences, healthy pigs treated with proteasome inhibitor showed hypertrophy and reduced heart function (Herrmann et al. 2013). In contrast, inhibiting proteolysis by PS-519 suppressed leukocyte infiltration, which diminished fibrotic deposition after myocardial infarction (Stansfield et al. 2007). Proteolysis blockage was reported to enhance cMyBP-C expression in hypertrophic cardiomyopathy (Carrier et al. 2009; Powell et al. 2012). Some components were connected with heart disease progression as in the case of the E3 ligase FBXO32, a homozygous mutation of this factor was associated with familial dilated cardiomyopathy (Al-Yacoub et al. 2016). Other E3 ligases, such as Atrogin-1, were presented to react with calcineurin and promote its degradation. Together, these evidence indicate that a proper balance of ubiquitination is crucial for heart function. Despite the controversial data about the actual roles, many studies indicate the dysfunction of ubiquitination in human cardiomyopathy, and its impairment in animal models supports this observation (Kumarapeli et al. 2005; Pagan et al. 2013; Spänig et al. 2019).

As discussed above, the ubiquitination machinery can be explored for

therapeutic approaches in cardiac diseases. For example, the MuRF family plays crucial roles in heart function, by which the deficiency of MuRF1 and MuRF3 protein in double mutant mice led to hypertrophic cardiomyopathy (Fielitz et al. 2007). The E3 complex Fbox32-MAFbx-Atrogin-1 mediates the susceptibility of cardiomyocytes in response to cardiac injuries through its impacts on apoptosis (Xie et al. 2009). Among the ubiquitination factors functioning negatively to the heart, cylindromatosis (CYLD) is one of the most characterised DUBs. It was reported to induce transdifferentiation of adventitial fibroblasts associated with vascular remodelling (Yu et al. 2017). In addition, CYLD deficient mice exhibited improved survival with less cardiac fibrosis and apoptosis in pressure overload condition (Wang et al. 2015a).

In conclusion, these information are concordant in indicating that targeting ubiquitination in the heart can possibly lead to the development of a novel therapeutic approach to combat heart disease.

2. AIM OF THESIS

The works described in this thesis is aimed at identifying the ubiquitination factors regulating cardiomyocyte proliferation. Using an unbiased High Throughput Screening approach, we performed a large scale of siRNA-based screening targeting the ubiquitination-proteasome pathway in neonatal mouse cardiomyocytes, followed by the analysis of cardiomyocyte proliferation using siRNAs against the identified top hits.

The screening led to the discovery of eleven top factors essential for cardiomyocyte replication. After that, we validated these factors and tested their capacity to enhance cardiomyocyte proliferation using an Adeno-Associated Virus (AAV) vector to overexpress these candidates in neonatal cardiomyocytes. These results led to the identification of an E2 conjugating enzyme named UBE2G1 induced significantly cardiomyocyte replication and thus was selected for further investigation.

We deepened our study by silencing this factor and evaluated its crucial role in cardiomyocyte replication in normal conditions and upon the stimulation of proliferation using a series of miRNAs that the laboratory had previously identified. We expanded our investigation on UBE2G1 by assessing whether this factor works through the ubiquitination cascade. To better understand the impact of UBE2G1 on cell replication, we examined the expression of cell cycle regulators and the activation of several signal transduction pathways.

Next, we validated the effects of UBE2G1 overexpression in enhancing cell replication in a neonatal mouse model. We also assessed its potential in protecting the heart after myocardial infarction in an adult mouse model.

Our findings provide new insights into UBE2G1 function on the proliferation of cardiomyocytes while proposing a further direction to investigate the UBE2G1 molecular mechanism in the heart.

3. MATERIALS AND METHODS

3.1 Cell culture methods

3.1.1. Human embryonic kidney 293T line (HEK293T)

HEK293T cells were purchased from ATCC and cultured in DMEM 1g/L glucose (Life Technologies), 10% foetal bovine serum (FBS) (Gibco) and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma).

3.1.2. Primary neonatal mouse ventricular cardiomyocytes

The hearts, harvested from 1-2 days old newborn mice, were transferred in ice-cold CBFHH buffer (calcium and bicarbonate-free Hanks with HEPES). Ventricles were separated from atria, and the tissue was minced into small pieces down to a diameter of 2-3 mm, digested with 0.5% Trypsin-EDTA solution (Life Technologies) at the final concentration 0.1 mL per mg of tissue in 4 minutes incubation at 37°C. The supernatant was collected in ice-cold FBS after each incubation. The digestion was repeated for 6-8 times. Cell suspension was filtered through a 40 μm strainer (BD Falcon) and centrifuged for 10 minutes at 1,500 rpm. The cells were resuspended in DMEM 4.5 g/L glucose, 10% FBS and pre-plated for 2 hours on 100 mm dishes to allow the fibroblast attachment. The supernatant containing mainly cardiomyocytes was then collected and plated on primary culture plates with DMEM 4.5 g/L glucose, 5% FBS, 20 mg/ml vitamin B12 (Sigma), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma).

3.1.3. Primary neonatal rat ventricular cardiomyocytes

After sacrificing neonatal Wistar rats (1-2 days old), the hearts were harvested and transferred in CBFHH buffer. The ventricles were separated from atria and the tissue was minced into small pieces down to a diameter of 2-3 mm and dissociated in CBFHH buffer containing 2 mg/ml of Trypsin, 20mg/ml of DNAse II and 80 ug/ml of Gentamicin. The digestion was performed in a fresh buffer for approximately 10 rounds of 10 minutes each

round with slow stirring at 37°C, followed by pipetting up and down. After each digestion, the supernatant was collected in warm FBS. At the end of digestion, cell suspension was filtered through a 40 µm strainer (BD Falcon) and centrifuged for 10 minutes at 1,500 rpm. The cells were resuspended in DMEM 4.5 g/L glucose, 10% FBS and pre-plated for 2 hours on 100 mm dishes to allow the fibroblast attachment. The cardiomyocytes, after this passage, were mainly present in the supernatant and easily collected for plating at the desired density in primary culture plates with DMEM 4.5 g/L glucose, 5% FBS, 20 mg/ml vitamin B12 (Sigma), 100 U/ml penicillin and 100mg/ml streptomycin (Sigma).

3.1.4. Plasmid cloning and AAV vector production

For AAV vector production, I had amplified the cDNAs of the selected factors PCR, then purified and cloned them into a plasmid backbone containing Flag-tag under the CMV promoter. Three-hundred µg of plasmid was used for AAV preparation. Vectors were generated by the AAV Vector Unit (AVU) at ICGEB Trieste (http://www.icgeb.org/avu-core-facility.html) as described previously with a few modifications (Arsic et al. 2004). Briefly, the infectious recombinant AAV vector particles were generated in HEK293T cells cultured in roller bottles by a cross-packaging approach whereby the vector genome was packaged into AAV capsid serotype 9 or AAV capsid 6. Viral stocks were obtained by PEG precipitation and $CsCl_2$ gradient centrifugation. The titer of recombinant AAVs was calculated by quantifying vector genomes (vg) packaged into viral particles, by using a real-time PCR calculated a standard curve of a plasmid containing the vector genome; the titer was in the range of $1x10^{12}$ to $1x10^{13}$ (vg/ml).

3.1.5 Primary neonatal cardiomyocytes transduction

After 24 hours of isolation, primary neonatal cardiomyocytes were incubated with AAV6 vectors at a multiplicity of infection (MOI) ranging from $2x10^4$ to $8x10^4$ viral genomes (vg) per cell.

3.1.6 siRNA/miRNA transfection on neonatal cardiomyocytes

The siRNA or miRNA mimics were transfected into a neonatal rat or neonatal mouse cardiomyocytes using either an optimised reverse or forward transfection protocols. The following procedures and volumes refer to a well of 96 multi-well plate. For each reaction, 0.2 µl of Lipofectamine RNAiMAX (ThermoFisher) were diluted in 25 µl of Opti-MEM Reduced Serum Medium (ThermoFisher) and incubated for 5 minutes before adding to 7.5 µl of siRNA or miRNA mimic (500 nM stock concentration, 25 nM final concentration). The transfection mixture was incubated for 30 minutes at room temperature and then transferred into the 96 multi-well plate. The medium was changed after 24 to 48 hours of transfection.

3.1.7 Cell cycle analysis

For flow-cytometric analysis of DNA content, the cardiomyocytes were harvested after 48 hours of transfection. The cells were fixed in 4% PFA for 15 minutes, permeabilized in 1% Triton X-100 in PBS for 20 minutes, blocked in 2% BSA (Roche) in PBS for 30 minutes, then stained with an antibody against sarcomeric α-actinin (ab90776, Abcam) in the blocking solution for 10 minutes and subsequently incubated with Hoechst 33342 (Life Technologies) for 10 minutes. After that, the cells were washed with PBS to remove the staining solution, centrifuged and resuspended in PBS. Cell suspensions were analyzed at the FACS Celesta Cytometer (BD). Analysis of the data was performed on FlowJo X software.

For the work described in this section, I would like to acknowledge the contribution of Ilaria Secco, PhD.

3.1.8 Immunofluorescence on primary cell cultures

To study cell cycle status of in vitro treated cardiomyocytes, we used the Click-IT technology to visualize the incorporation of the thymidine analogue EdU, added to the cell culture medium at 20 hours before the fixation. The cardiomyocytes were separated from the containing non-myocyte cells by a specific staining with cardiac sarcomeric α-actinin. Briefly, primary

cardiomyocytes were fixed with 4% PFA for 10 minutes, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, followed by 30 minutes blocking in 1% BSA in PBS. The cells were stained overnight with an antibody against sarcomeric α-actinin diluted in blocking solution at 4°C. After that, the cells were washed with 0.2% Tween-20 in PBS and incubated for 2 hours with relevant secondary antibodies conjugated to Alexa Fluor Dyes (LifeTechnologies). Eventually, the cells were stained by the Click-IT EdU 594 Imaging kit (Life Technologies) to detect EdU incorporation, according to the manufacturer's instructions, followed by the final staining with Hoechst 33342.

3.2 High Throughput Screening

The work described in this section was performed with the technical assistance of Luca Braga, PhD and the supervision of Hashim Ali, PhD.

A murine library of more than 600 siRNAs of known ubiquitination factors (Dharmacon) was used to transfect in neonatal mouse cardiomyocytes. Using a liquid handler (Hamilton) to dilute siRNAs from the library, 5 µl of siRNA (500nM stock concentration) was pipetted into each well of a 384-wells plate (Perkin Elmer), leaving space for controls on either side. A pre-incubated mixture of RNAiMAX and Opti-MEM was added to each well using a multi-well dispenser and incubated for 30 minutes. The suspension containing isolated cardiomyocytes was added to each well in DMEM 4.5 g/L glucose, 5% FBS, 20 mg/ml vitamin B12 without antibiotics. After 72 hours transfection, EdU was added in the culture medium, and the cells were fixed after 20 hours of incubation. Immunofluorescence was performed as previously described to exclude the contribution of contaminating fibroblast from the quantification.

High content fluorescent images were acquired using the ImageXpress micro microscope (Molecular Devices) and analysed using MetaXpress. A total of 12 images were acquired per well for each wavelength.

Cardiomyocytes were detected in the primary cultures by their positivity for sarcomeric α-actinin.

3.3 Molecular biology methods

3.3.1 Generation of the plasmid collection

The coding sequence of selective gene was amplified through PCR from extraction samples of the mouse heart. Gel extraction and PCR clean up were performed using Wizard SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions. After that, the amplicon was cloned directly into pAAV-CMV plasmid available in the AAV Unit. The plasmid was transformed into XL10-Gold ultracompetent cells (Stratagene) and prepared using NucleoBond Xtra Midi or Maxi EF kit (Macherey-Nagel) following the manufacturer's instruction. Plasmid sequencing was done by GATC Services (Eurofins Genomics). For evaluating of protein expression, cloned plasmid was transfected in HEK293T and examined with western blotting. These plasmids were used for AAV vector preparations.

Primers for cloning (5'-3'):

- *Otud7b*	Fwd: ATGACCCTGGACATGGATGCTG
	Rev: TCAGAACCTGTGTGCCAGCAGT
- *Phf5a*	Fwd: ATGGCTAAACATCATCCAGA
	Rev: TCACCTCTTCTTGAAGCCGTATT
- *Prpf19*	Fwd: ATGTCCCTGATCTGCTCGATCT
	Rev: TACAGACTGTAGAATTTGAGGCT
- *Socs5*	Fwd: ATGGATAAAGTGGGGAAAATGTG
	Rev: TACTTTGCTTTGACTGGTTCTCG
- *Ube2g1*	Fwd: ATGACGGAGCTGCAGTCGGCGC
	Rev: TCACTCAAAAGCAGTTTCTTGGCTT
- *Usp36*	Fwd: ATGCCTATAGTCGATAAGCTGAAGG
	Rev: TCAGCGGCGATAGCTGAGGCTG
- *Dcund1d3*	Fwd: ATGGGCCAGTGTGTCACCAA
	Rev: CTAAGTCTGCTCTTCTGGAC
- *Rbck1*	Fwd: ATGGACGAGAAGACCAAGAA

Rev: TTAGTGGCAGTTTTGACAGC

- *Rnf181* Fwd: ATGGCGTCTTATTTTGATGAGCA
  Rev: TCACGTGTACATGGCTCCGT
- *Rnf186* Fwd: ATGCCAGACATCGGTTGTCAGCA
  Rev: TCAGGCAATAGAAGTGATCTGGGTT
- *Ube2e1* Fwd: ATGTCGGATGACGATTCGAGGG
  Rev: TTATGTAGCGTATCTCTTGGT

3.3.2 RNA isolation and quantitative real-time PCR

Total RNA was extracted by using QIAzol (QIAGEN) following the manufacturer's instruction. RNA quantification was carried out by using Qubit 2.0 Fluorometer (Invitrogen). An amount of 0.5-1 µg of the total RNA was reverse transcribed using Random primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen). All the quantitative real-time PCR were performed on a BioRad CFX96 using TaqMan probes (Applied Biosystem) or SYBR (Promega). A dissociation curve was used to confirm primer specificity when performing experiments with GoTaq.

TaqMan assays:

- *Gapdh* Mm99999915_g1
- *Hprt* Mm01545399_m1
- *Ube2g1* Mm00482548_m1
- *Notch1* Mm00627185_m1
- *Hey1* Mm00468865_m1
- *Hes1* Mm01342805_m1
- *CyR61* Mm00487499_m1
- *Ctgf* Mm01192933_g1

Primers for quantitative real-time PCR with SYBR:

- *p15* Fwd: CCCGATCCAGGTCATGATGA
  Rev: CATTAGCGTGTCCAGGAAGC
- *p16* Fwd: CGGTATCTACTCTCCTCCGC
  Rev: GTTGCCAGAAGTGAAGCCAA
- *p21* Fwd: CCTCCCAGCCTCCAAACTTA

	Rev: CGCCAGGATCAGAAACACAG
- *p27*	Fwd: TTGGGTCTCAGGCAAACTCT
	Rev: TCTTCTGTTCTGTTGGCCCT
- *Cyclin D1*	Fwd: AGAAGTGCGAAGAGGAGGTC
	Rev: CTTAGAGGCCACGAACATGC
- *Cyclin E*	Fwd: CTGTCAGCTGACAGTGGAGAAGG
	Rev: AGGGTGCTACTTGACCCACTGGA
- *Anf*	Fwd: ATACAGTGCGGTGTCCAACA
	Rev: CGAGAGCACCTCCATCTCTC
- *Gapdh*	Fwd: CTGTCAGCTGACAGTGGAGAAGG
	Rev: AGGGTGCTACTTGACCCACTGGA
- *Hprt*	Fwd: CTGTCAGCTGACAGTGGAGAAGG
	Rev: AGGGTGCTACTTGACCCACTGGA

3.3.3 Western blotting

To assess the protein levels of several proteins, samples were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.2; 150 mM NaCl; 1% Triton X-100) complemented with Protease Inhibitor Cocktail (Roche) for 30 minutes at 4°C. Protein quantification was done by Bradford assay (BioRad). The samples were resuspended with Laemmli sample buffer (0.19 M Tris-HCl, pH 6.8; 0.015% bromophenol blue; 30% glycerol; 3% SDS; 3% 2-mercaptoethanol) and denatured by boiling for 5 minutes at 90°C. Then, they were resolved by SDS-PAGE of the relevant percentage according to the protein of interest. Total proteins were transferred at 350 mA for 90 minutes at room temperature onto nitrocellulose membrane (GE Healthcare) and then blocked in 5% milk TBST for 1 hour at room temperature. The membrane was incubated overnight with primary antibody at 4°C. After that, the membrane was washed with TBST for 3 times and incubated with secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature. Following the membrane development, the band intensity was analysed using ImageJ software.

The following antibodies were used in this study:

- Flag M2-HRP	A8592, Sigma-Aldrich
- β-Actin-HRP	A3854, Sigma-Aldrich
- UBE2G1	PA5-30201, Thermo Fisher
- CDT1	sc-365305, Santa Cruz
- pERK1/2	9106, Cell Signaling
- Cleaved Notch1 (Val1744)	BK2421L, Cell Signaling
- YAP	4912S, Cell Signaling
- pYAP (S127)	4911S, Cell Signaling
- Tubulin	T5168, Sigma-Aldrich

3.3.4 Immunoprecipitation

HEK293T cells were transfected with the plasmids using FuGENE HD transfection reagent (Promega). After 48 to 72 hours of transfection, cells were washed with cold PBS and lysed with RIPA buffer for 30 minutes at 4°C. Samples were then sonicated and quantified with Bradford assay (BioRad). For one round of IP, approximately 0.5-1 mg total protein was incubated with pre-equilibrated anti-Flag M2 magnetic beads or dynabeads pre-conjugated with target antibodies on a rotating wheel at 4°C for 2 hours. Then, the beads were washed 5 times with cold lysis buffer, resolved in Laemmli sample buffer and examined by western blotting with specific antibodies.

3.3.5 Silver staining

After resolving samples by SDS-PAGE, the gels were placed in fixative solution (40% ethanol, 10% acetic acid) for 20 minutes and subsequently stained with SilverQuest™ Silver Staining Kit (Life Technologies) following manufacturer's instruction.

3.3.6 Proteome Profiler Array

After 48 hours of transfection or transduction, cardiomyocytes were treated with 10 μM of proteasome inhibitor MG132 (Calbiochem) for 2 hours. Then, the samples were washed with cold PBS and lysed with lysis buffer (supplied by the kit and complemented with 10 μg/mL Aprotinin, 10

μg/mL Leupeptin and 10 μg/mL Pepstatin) for 30 minutes at 4°C. The lysates were centrifuged at 14,000g for 5 minutes and transferred into a clean tube. After the quantification, around 250 μg of total protein lysates were used for the next step following the manufacturer's instruction. In brief, the cell lysates were incubated at 4°C overnight with the membranes (supplied by the kit) on a shaker. Then, the membranes were washed for 3 times with the washing buffer (supplied by the kit), and incubated with the detection antibody cocktail (supplied by the kit) at room temperature for 2 hours. After the incubation, the membranes were washed for 3 times with the washing buffer and subsequently incubated for 30 minutes with the Streptavidin-HRP (supplied by the kit). The membranes were eventually washed and developed with Chemi Reagent Mix (supplied by the kit).

3.3.7 Nuclear-cytoplasmic fractionation

After 72 hours of transfection or transduction, cardiomyocytes were washed with cold PBS and harvested in 100 μl of hypotonic buffer (50 mM Tris pH7.5, 10 mM NaCl, 3 mM MgCl2, 10% glycerol) supplemented with protease inhibitors (Roche) and phosphatase inhibitors (cocktail 3 Sigma, 2mM orthovanadate, 1 mM NaF). After 1 minute incubation, the cells were collected and NP40 was added to 0.1% final concentration. After 5 minutes on ice, the cells were centrifuged at 4,000 rpm for 5 minutes at 4°C; the supernatant and cytosolic fractions were then recovered. The pellets were resuspended in 150 μl IPLS buffer (50 mM Tris pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.5% NP40, supplemented with the same inhibitors as above) and sonicated. Nuclear lysates were centrifuged at 16,000rpm for 20 minutes at 4°C and the supernatant was recovered as the nuclear fraction. Protein concentration of cytoplasmic and nuclear lysates was measured using the Bradford assay (BioRad). A total of 25 μg protein lysates were used for western blotting onto a nitrocellulose membrane (GE Healthcare).

3.4 Animal experimentations

Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (European and Economic Council Directive 86/609, OJL 358, December 12, 1987), upon approval by the ICGEB Institutional Animal Welfare Board and by the Italian Ministry of Health.

Among the procedures described in this section, echocardiography, intracardiac injection and myocardial infarction were performed by Simone Vodret, PhD.

3.4.1 Intraperitoneal injection in neonatal mice

Postnatal CD1 mice (P0-P1) received roughly 30 µl of injection solution intraperitoneal by Microfine Plus 0.3 ml Insulin Syringe 8 mm/30 gauge (BD). Every mouse received approximately $3x10^{11}$ AAV9 vectors. Following day, EdU was injected in the mice with dosage adjusted by animal body weight at 30 g/kg final concentration. Edu injection was repeated every other day for 7 days.

3.4.2 Intracardiac injection in adult mice

Adult CD1 female mice were anesthetized by intraperitoneal injection of ketamine (40 µg/g, Imalgene 1000) and xylazine (100 µg/g, Sigma), at a dosage of 1.2 to 1.3 µl/g and intubated. Thorax was incised, the fifth intercostal space was cut and enlarged to expose heart anterior wall. Maximum 30 µl of AAV9 suspension (ranging from $6x10^{10}$ to $3x10^{11}$) was injected into the left ventricle. After injection, the thorax was sutured and mice were extubated to re-establish normal breathing.

3.4.3 Myocardial infarction

Myocardial infarction was introduced in adult CD1 mice (8 to 12 weeks old) by permanent left anterior descending (LAD) coronary artery ligation. The mice were anaesthetised on a warming pad (37°C) and endotracheally intubated on a rodent ventilator. Beating heart was accessed via a left

thoracotomy. After removing the pericardium, a descending branch of the LAD coronary artery was visualised with a stereomicroscope (Leica) and occluded with a nylon suture. Ligation was confirmed by the whitening partly of the left ventricle, immediately post-ligation. After that, the mice received an intracardiac injection of AAV9 vectors in the left anterior infarct border zone, as described above. The chest was then closed, and the mice were kept on the warm pad until they recovered spontaneous breathing.

3.4.4 Echocardiography

Trans-thoracic two-dimensional echocardiography was performed at 15 days, 30 days and 60 days after myocardial infarction. The mice were sedated with 5% isoflurane, using the Vevo 770 Ultrasound (VisualSonics), equipped with a 30-MHz linear array transducer. M-mode tracing was used to measure left ventricular anterior and posterior wall thickness, internal diameter at end-systole and end-diastole, fractional shortening and ejection fraction.

3.4.5 Trichrome staining

At the end of the studies, the heart was excised, briefly washed in PBS, and fixed in 10% formalin overnight at room temperature to proceed the embedded paraffin section and used for histology or immunofluorescence staining. Masson's trichrome was used to stain the keratin and muscle fibers in red, collagen and bone in blue, cytoplasm in pink, and cell nuclei in dark brown to black. Tissue paraffin sections were deparaffinized (65°C for 12 hours and xylene or bioclear for 2 hours at room temperature), rehydrated (5 minutes in 100% EtOH, 5 minutes in 95% EtOH, 5 minutes in 75% EtOH, 5 minutes in 50% EtOH, and 5 minutes in water) and processed using a commercially available kit for Masson's trichrome stain (BioOptica), according to the manufacturer's instruction. Later, the slides were rapidly dehydrated and mounted in Eukitt mounting medium (Sigma).

3.4.6 Immunofluorescence in paraffin-embedded tissue

Four-µm thick tissue sections were de-waxed and rehydrated. Antigen retrieval was performed by boiling the sections in 10 mM sodium citrate buffer pH 6.0, 0.05% Tween-20 for 20 minutes and letting them cool down at room temperature for 2 hours. Sections were rinsed three times in water and permeabilised for 30 minutes in 0.5% Triton X-100 in PBS, then blocked for 1 hour in 2% BSA in PBS. Tissue sections were stained overnight at 4°C with an antibody against sarcomeric α-actinin. After that, the slides were washed in 0.05% Tween-20 in PBS, incubated for 2 hours with secondary antibody conjugated to Alexa Fluor Dyes (LifeTechnologies). Samples were further processed using the Click-IT EdU 594 Imaging kit and stained with Hoechst 33342.

3.5 Statistical analysis

Statistical analysis was performed by using a commercial software package (GraphPad Prism). Data are presented as mean ± standard error of the mean (s.e.m.). The comparison between two groups was performed by the Student's t-test, that among different groups with one-way ANOVA or two-way ANOVA followed by post-hoc analysis using the Bonferroni's method. The levels of statical significance was set at $p<0.05$.

4. RESULTS

4.1 The identification of novel ubiquitination factors involved in cardiomyocyte proliferation

Cardiomyocyte proliferation is essential for heart regeneration. So far, numerous studies reported attempts to reactivate the cardiac regenerative capacity. However, none of these studies tackled the role of ubiquitination in cardiomyocyte proliferation. Therefore, we decided to investigate the possible therapeutic role of ubiquitination in heart disease.

A high throughput screening (HTS) approach to identify cardiomyocyte regulators was performed successfully in the Molecular Medicine Laboratory (Eulalio et al. 2012). In this study, we exploited HTS to test a library of ubiquitination factors involved in cell replication. Our synthetic murine siRNA library includes more than 600 known members of ubiquitination (four different siRNAs for each gene). This library was used for reverse transfection in primary neonatal cardiomyocytes, and a proliferation assay based on 5-ethynyl-29-deoxyuridine (Edu) incorporation was implemented as a major readout after 72 hours (**Figure 4.1A**) (Edu is a modified thymidine analog that becomes incorporated in newly synthesized DNA). miR-199-3p and miR-590-3p were used as positive controls of proliferation. The screening was performed in duplicate, exhibiting good reproducibility between the two replicates (Pearson $R=0.6104$, $p<0.0001$). We assessed cell viability by counting the total cell number and evaluated the data distribution with the Shapiro-Wilk test.

We observed that 592 siRNAs did not hamper cell viability, while the siRNAs exerting a toxic effect were excluded from further investigation. The results are shown in **Figure 4.1B** and are presented as $\log_2$ fold of cardiomyocyte proliferation of treated samples over controls. The silencing of several factors led to a significant decrease in cardiomyocyte proliferation, indicating an essential role of ubiquitination in this process

(**Figure 4.1C**). In parallel, knockdown of some factors resulted in an increase in cardiomyocyte proliferation. In this thesis, we focused mainly on the factors required for cell cycle progression.

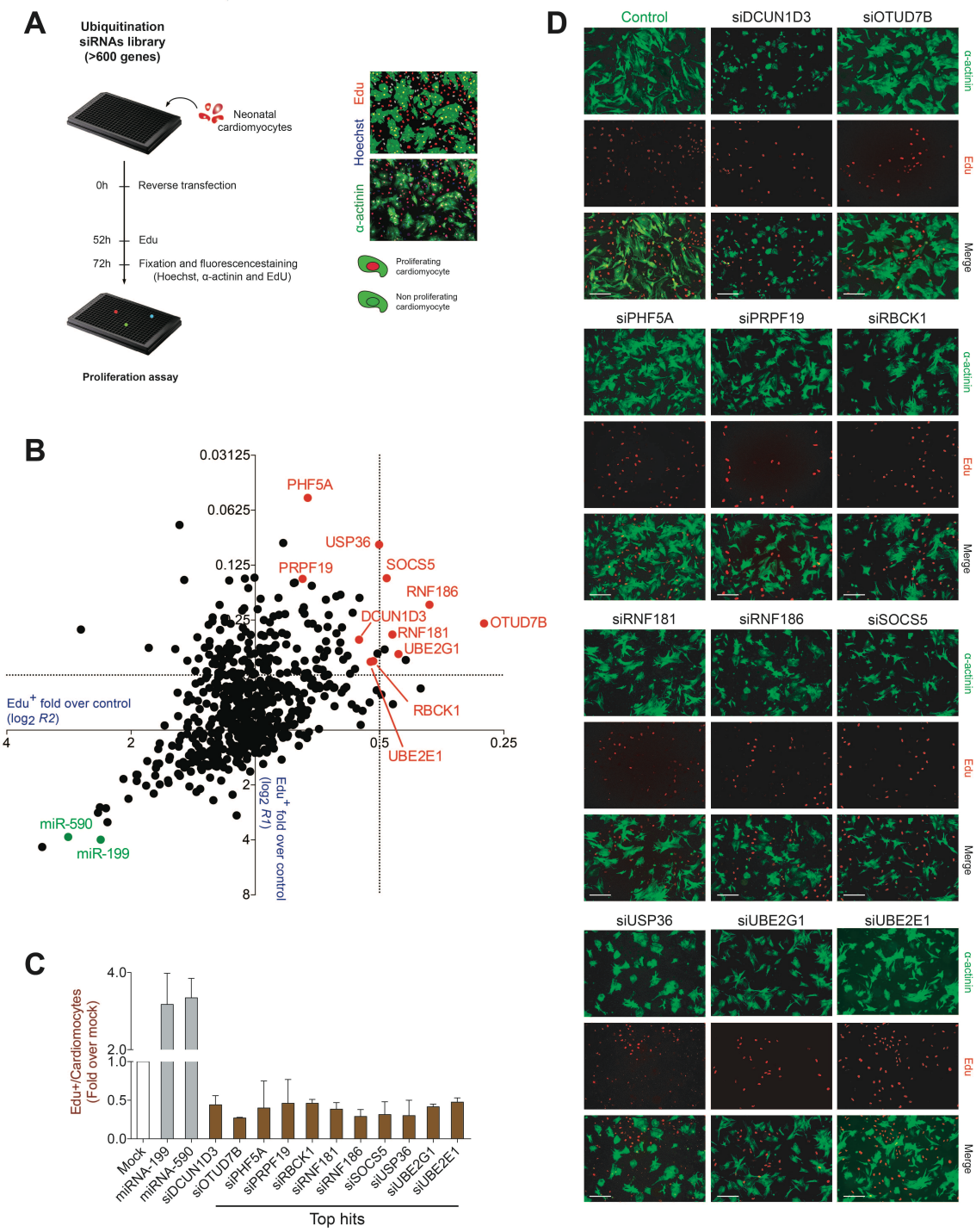

Figure 4.1: High throughput siRNA-based screening identifies ubiquitination factors involved in cardiomyocyte proliferation. A. Schematic of the screening and image reconstruction for quantification of proliferating cardiomyocytes. **B.** Screening results; the graph shows $\log_2$ fold of proliferative cardiomyocytes over control in two

replicate screenings (R1 and R2, Pearson R=0.6104, p<0.0001); the dotted lines indicate a 2-fold decrease of proliferation compared with control, selective factors are in red, miR-199-3p and miR-590-3p are in green. **C.** Cardiomyocyte proliferation of 11 top factors and controls expressed as fold change over untreated cardiomyocytes, data are mean ± s.e.m (n = 3 independent experiments). **D.** Representative images of cardiomyocytes treated with siRNAs against selected factors and miRNA controls stained with Edu and α-actinin. Scale bars 100 μm.

Eleven genes were selected for further analysis, including two E2 conjugating enzymes (UBE2G1, UBE2E1), two deubiquitinating enzymes (OTUD7B, USP36), and seven E3 ligases (DCUN1D3, PHF5A, PRPF19, RBCK1, RNF181, RNF186, SOCS5) (**Figure 4.1C**). The selection of PHF5A was considered as a good indication of the efficacy of the screening since depletion of PHF5A was already reported to reduce the self-renewal potency of embryonic stem cells (Strikoudis et al. 2016). Maintenance of these genes appears to be essential for basal cell proliferation, thus we asked whether their overexpression might enhance cardiomyocyte replication. To answer this question, we generated the corresponding adeno-associated virus (AAV) to deliver them into primary cells.

4.2 *In vitro* validation of the proliferative effect of the top factors

To efficiently deliver the genes of interest in cardiomyocytes, we exploited the cardiac tropism and long-term expression of AAV6. We cloned the coding sequences of these genes into a plasmid AAV backbone including a Flag-tag and under the control of a CMV promoter. The cloning was assessed by sequencing and western blotting (**Figure 4.2A**).

Next, we isolated neonatal cardiomyocytes and incubated them with AAV6 vectors expressing target proteins at the multiplicity of infection (MOI) of $5x10^4$ (vg/cell) for 72 hours. This MOI was used as described in the previous works of our laboratory (Lovric et al. 2012; Ruozi et al. 2015). AAV6-Flag and miR-199 served as empty control and positive control of proliferation, respectively. The readout of this proliferation assay was Edu

incorporation into cardiomyocyte DNA (**Figure 4.2B**), and the experiment was performed in duplicate.

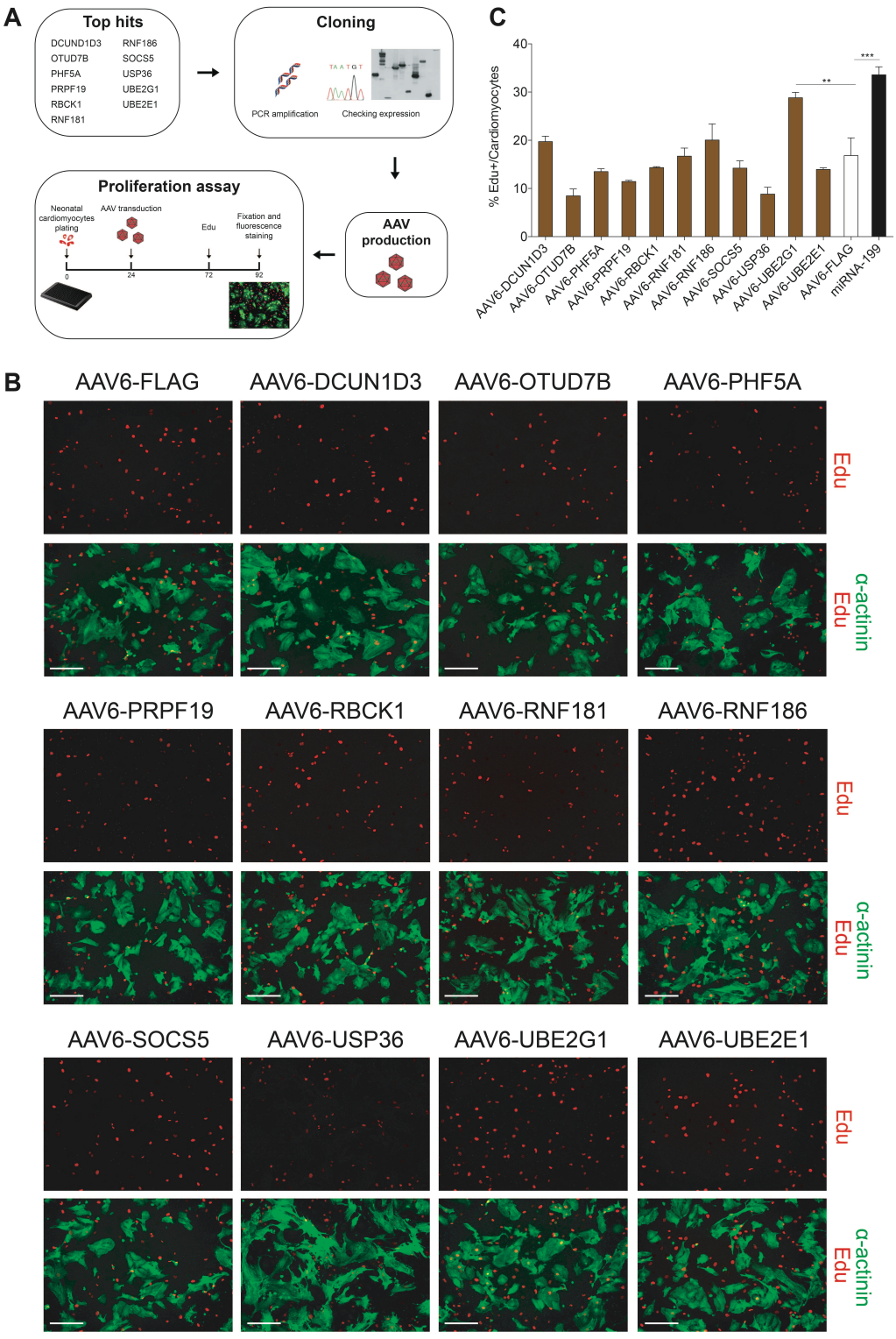

Figure 4.2: *In vitro* evaluation of the proliferative effect of selective factors. A. Experimental workflow; the top hits were cloned into an AAV vector fused to a Flag-tag; protein expression was checked by western blotting, individual AAV6 was

produced and used for proliferation assay. **B.** Representative images of cardiomyocytes stained with Edu and α-actinin after AAV transduction (alpha-actinin, green, Edu, red). Scale bars 100 μm. **C.** Percentage of proliferating cardiomyocytes after AAV6 transduction, data are mean ± s.e.m (n = 3 independent experiments), *p<0.05, **p<0.01, one-way ANOVA.

We observed that the overexpression of a few genes was able to increase cardiomyocyte proliferation. In particular, the E2 conjugating enzyme UBE2G1 was the most significant factor in enhancing proliferation (**Figure 4.2C**). Therefore, we decided to further focus our attention on UBE2G1.

4.3 The depletion of UBE2G1 abolished the effect of pro-proliferative miRNAs and induced hypertrophy

After selecting UBE2G1, we investigated its role in cardiomyocyte function. Since the screening was performed using a pool of 4 siRNAs, we individually tested the four separate deconvoluted siRNAs. We found that siUBE2G1-3 was the most effective siRNA (**Figure 4.3A**) and used it in the following experiments. This experiment was performed only once due to the limited amounts of siRNAs cherrypicked from the screening library. After that, we validated the efficacy of silencing and overexpression of UBE2G1 at the mRNA and protein level. The results showed that siRNA treatment reduced the UBE2G1 mRNA level of 60%, while the AAV-mediated overexpression strongly induced both mRNA and protein expression of UBE2G1 (**Figure 4.3B and C**).

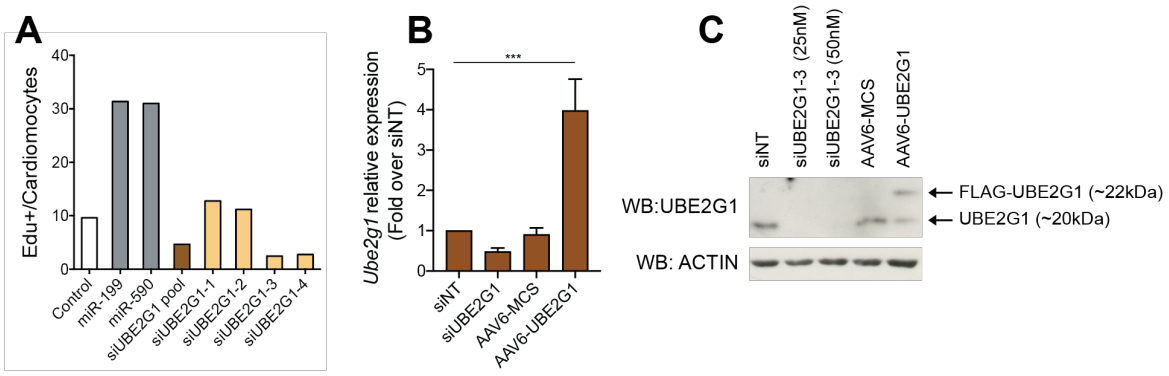

Figure 4.3: Validation of silencing and overexpression of UBE2G1. A.

Cardiomyocyte proliferation after transfection with a single siRNA or a pool of four siRNAs against UBE2G1. **B, C.** UBE2G1 expression measured by qPCR and western blotting, data are mean ± s.e.m (n = 3 independent experiments), **p<0.01, one-way ANOVA.

To confirm that UBE2G1 depletion blocked cardiomyocyte cell cycle progression, we also performed a Fluorescence Activated Cell Sorting (FACS) experiment. The data indicated that UBE2G1 silencing arrested cardiomyocytes significantly in the G1 phase compared with controls, subsequently reducing of 25% and 50% the cell numbers in S and G2/M phases, respectively (**Figure 4.4A**).

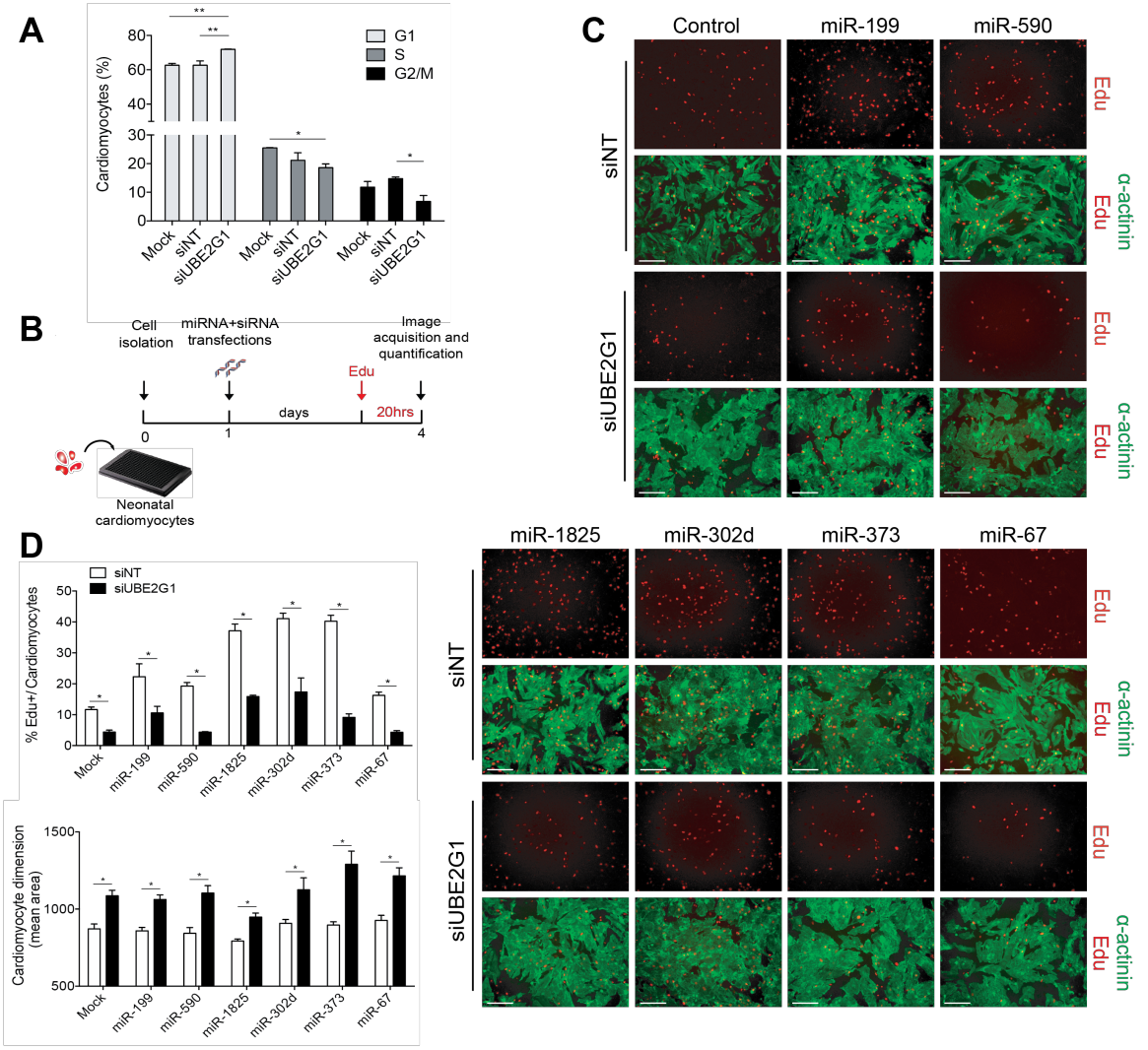

Figure 4.4: The depletion of UBE2G1 abolished the pro-proliferative effect of miRNAs and induced hypertrophy. A. FACS analysis of cardiomyocyte cell cycle, data obtained from quantification of at least 1.5x10^4 cardiomyocytes for each condition, data are mean ± s.e.m (n = 3 independent experiments), *p<0.05, **p<0.01,

two-way ANOVA. **B.** Experimental flow chart of siUBE2G1 and miRNA co-transfection. **C.** Representative images of cardiomyocytes stained with Edu and α-actinin after transfection. Scale bars 100 μm. **D.** Percentage of cardiomyocyte proliferation after siUBE2G1 and miRNA transfections (top panel), cardiomyocyte cross-sectional area after siUBE2G1 and miRNA transfections (bottom panel). Data are mean ± s.e.m (n = 3 independent experiments) *p<0.05, **p<0.01, t-test.

Following this observation, we asked whether UBE2G1 was essential for the proliferation of cardiomyocytes after a strong stimulation to enter the cell cycle. Our laboratory had previously identified several miRNAs inducing cardiomyocyte proliferation, such as miR-199-3p and miR-590-3p, acting through different pathways (Eulalio et al. 2012; Torrini et al. 2019). Therefore, we decided to investigate the possible connection of UBE2G1 and the pathways modulated by the pro-proliferative miRNAs. siRNA non-targeting (siNT) and siUBE2G1 were co-transfected into neonatal cardiomyocytes with human miR-199a-3p, miR-590-3p, miR-302c, miR-373, and miR-1825 for 72 hours. C. elegans miR-67 served as a control in this experiment. Proliferation was assessed by EdU incorporation as previously described (**Figure 4.4B**). We observed that the depletion of UBE2G1 dramatically suppressed the pro-proliferative effects of all tested miRNAs (**Figure 4.4C and 4.4D**). Notably, we also observed that siUBE2G1 impacted the cardiomyocyte phenotype (**Figure 4.4C and 4.4D**). Indeed, silencing UBE2G1 determined a significant increase in cardiomyocyte cell size and this hypertrophic response was also observed after treatment with all the tested miRNAs.

In summary, these results underlined the importance of UBE2G1 to cardiomyocyte cycling.

4.4 UBE2G1 acts through ubiquitination to modulate cardiomyocyte proliferation

UBE2G1 is an E2 conjugating enzyme consisting of a putative E2 domain with one cysteine in the active site and it does not require an N-terminus nor a C-terminus domain for the catalysis (Polge et al. 2015). To better

understand if the effects of UBE2G1 on proliferation was mediated by the ubiquitination pathway, we generated a UBE2G1mutant by substituting the cysteine in the active site with alanine (**Figure 4.5A**). After that, we generated the corresponding AAV6 vector for the mutant protein (UBE2G1-CA) and assessed its impact on proliferation. We found that AAV6-UBE2G1-CA significantly reduced cardiomyocyte replication in a dose-dependent manner (**Figure 4.5B and C**). Moreover, a slight, but statistically significant, increase in cardiomyocyte cross-sectional area was also observed by this treatment, similarly to the siUBE2G1, suggesting a dominant-negative effect exhibited by the mutated UBE2G1 (**Figure 4.5C, bottom panel**).

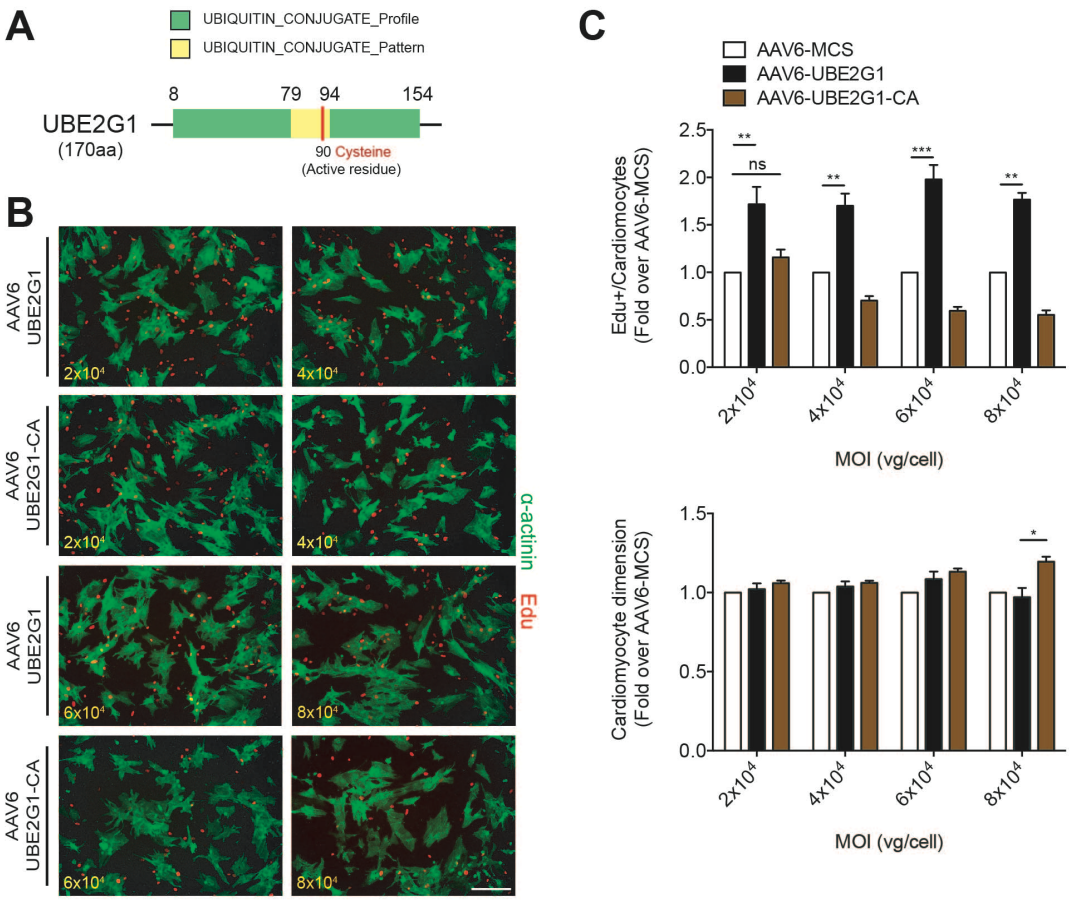

Figure 4.5: Mutation of the UBE2G1 catalytic site eliminates its proliferative effect. A. The scheme displays the UBE2G1 protein domains. The mutation of the catalytic site (Cysteine replaced by alanine, C>A) was introduced at the position in red. **B.** Representative images of proliferative cardiomyocytes after transduction with

increasingly higher MOIs of AAV6-UBE2G1, AAV6-UBE2G1-CA and AAV6-MCS, which was used as a control. Scale bar 100 µm. **C.** Percentage of cardiomyocyte proliferation after transduction (top panel), cardiomyocyte cross-sectional area after transduction (bottom panel); data are mean ± s.e.m (n = 3 independent experiments); *p<0.05, **p<0.01, two-way ANOVA.

Altogether, these data demonstrate that UBE2G1 triggers proliferation through ubiquitination and that a mutation in the active site abrogates this effect.

4.5 Identification of UBE2G1 partners

Since we observed that UBE2G1 acts through ubiquitination, we wanted to define its targets. UBE2G1 was previously associated with the E3 complex CRL1^{Skp1} for the regulation of CDT1 stability in human colon cancer cells. CDT1 is a well-known factor modulating DNA replication (Shibata et al. 2011; Pozo and Cook 2016). Thus, we wanted to evaluate whether UBE2G1 might function through CDT1 in the cardiac context (**Figure 4.6A**).

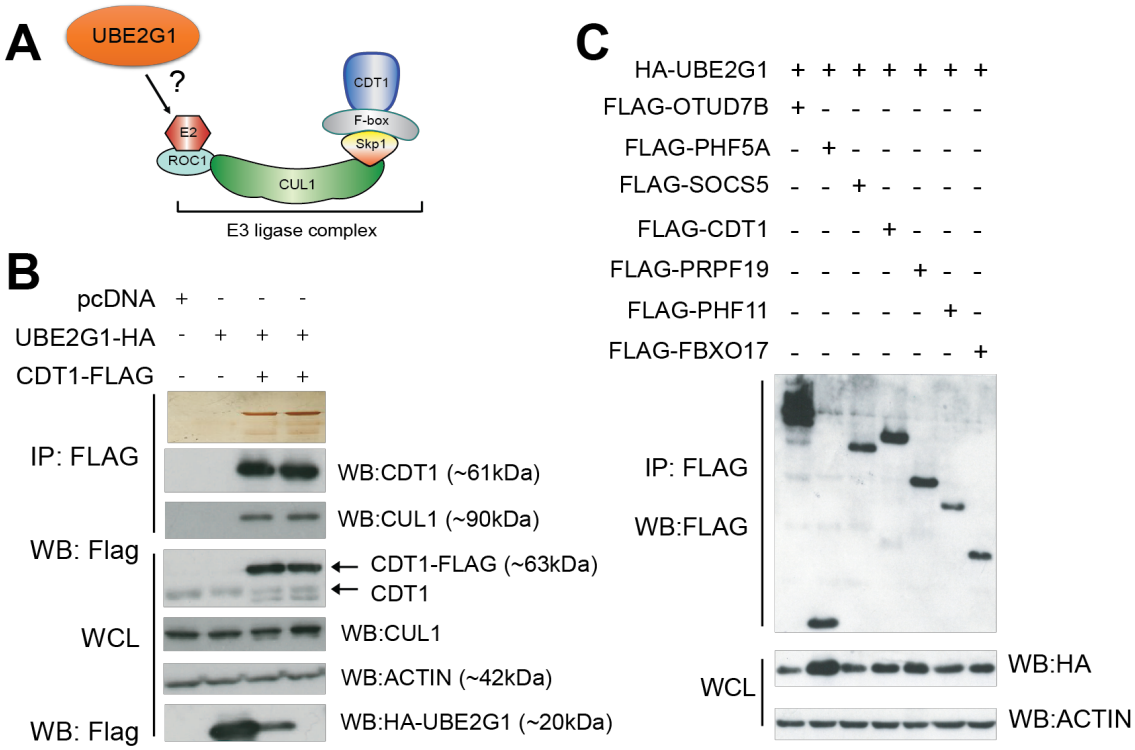

Figure 4.6: Investigation of possible interaction between UBE2G1 and E3 ligases. A. Scheme displays the possible connection of UBE2G1 and CRL1^{Skp1} complex to regulate CDT1 stability. **B.** Western blot results after immunoprecipitation

to detect the interaction of UBE2G1 and CDT1. **C.** Western blot results followed immunoprecipitation to detect the interaction of UBE2G1 with different E3 ligases.

Immunoprecipitation was applied to pulldown CDT1 and its interacting partners. As mentioned in the literature, we were able to detect an interaction between CDT1 and CUL1, but could not observe any binding between UBE2G1 and CDT1 in this setting (**Figure 4.6B**).

We decided to examine further a possible connection between UBE2G1 and the ligases discovered in the screening as potential modulators of cell cycle progression. We co-transfected the UBE2G1 plasmid with the indicated ligase into HEK-293 cells and then immunoprecipitated them using Flag beads. Unfortunately, we could not detect any interaction (**Figure 4.6C**).

4.6 The interplay of UBE2G1 with various modulators to regulate proliferation

As previously shown, the silencing of UBE2G1 blocks proliferation. Therefore, we wanted to investigate which cell cycle regulators were affected by UBE2G1 silencing. To answer this question, we examined the mRNA and protein levels of several cell cycle regulators.

Upon treatment with siUBE2G1, we found that p27 expression was significantly induced, looking at both mRNA and protein levels (**Figure 4.7A**). In parallel, the reduction of Cyclin D1, Cyclin E, and p15 expression was confirmed by quantitative PCR (**Figure 4.7A**). We could not detect Cyclin D and p15 proteins with our antibodies.

To better investigate additional pathways involved in UBE2G1 action, we analysed a commercial phosphorylation profile of cardiomyocytes upon overexpression and silencing of UBE2G1. Forty-three common kinases were tested. We found that the phosphorylation of GSK-3β, ERK1/2, and STAT3 proteins was significantly altered following our treatments (**Figure**

4.7B and C), suggesting a possible interaction of UBE2G1 with these pathways to allow cell cycle progression.

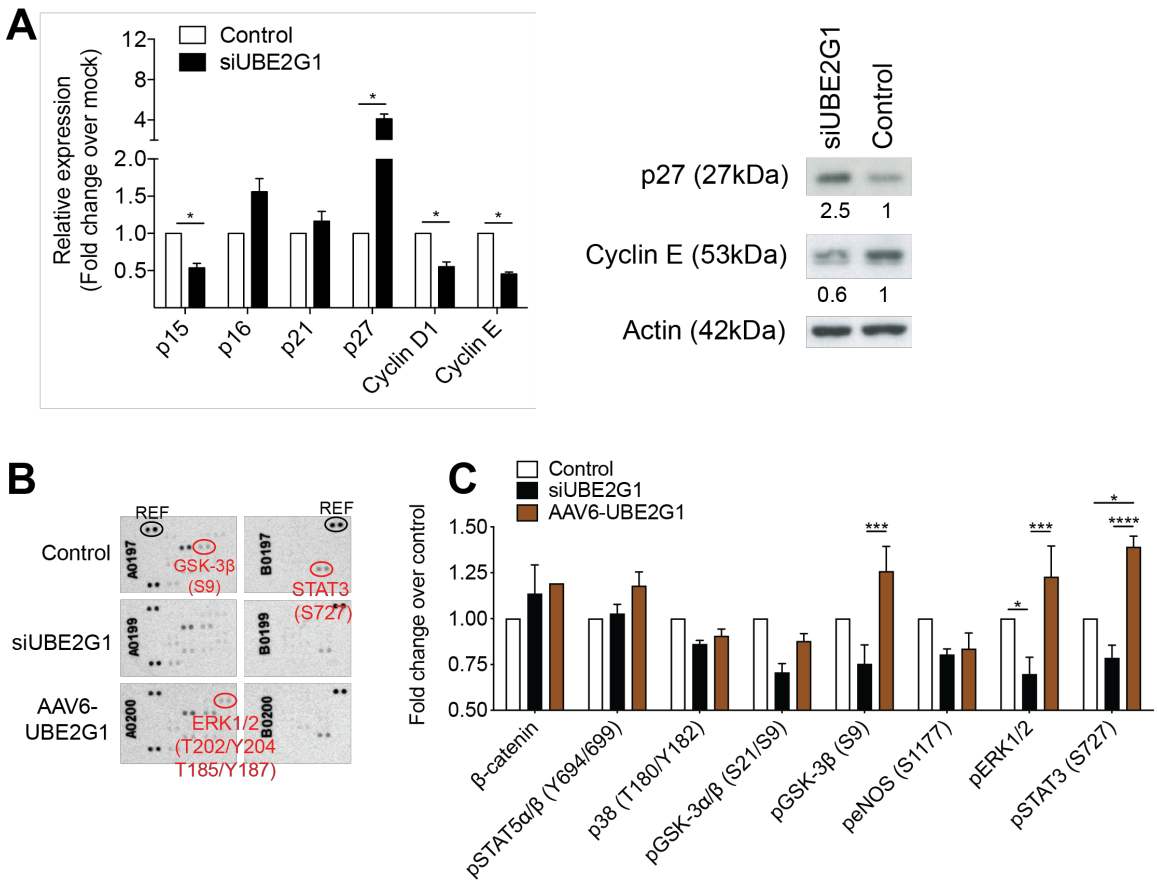

Figure 4.7: UBE2G1 affected cell cycle regulators and different kinases. A. Depletion of UBE2G1 affects the transcriptional levels of *p15*, *p27*, *Cyclin D1*, *cyclin E* and the protein levels of p27 and Cyclin E. **B.** Representative blots after the development of proteome profiler phospho-kinase array, reference spots are in black; **C.** Quantification of samples treated with siUBE2G1 or AAV6-UBE2G1 normalized by untreated sample, obtained as in (B). Data are mean ± s.e.m (n = 3 independent experiments), *p<0.05, **p<0.01, two-way ANOVA.

A few essential signalling pathways known to regulate cardiomyocyte proliferation were not included in the previous commercial panel. In particular, we wondered whether the master regulators of cardiomyocyte proliferation Notch and Hippo pathways were connected with the UBE2G1 mechanism of action. To clarify this question, we performed a nuclear and cytoplasmic fractionation experiment to assess the protein levels of the Notch1 intracellular domain (NICD), phosphorylated YAP (Serine 127), and

total YAP. The p84 protein and Tubulin levels were used as controls of the fractionation procedure. We observed a reduction of the NICD protein level in the nucleus upon UBE2G1 silencing, and the opposite effect after its overexpression (**Figure 4.8A**). These observations are consistent with the known role of NICD in regulating neonatal cardiomyocyte proliferation, not necessarily implying a direct function of UBE2G1 in the Notch1 pathway. On the YAP side, upon UBE2G1 overexpression, we observed a reduction of YAP phosphorylation at Serine 127, which promoted YAP degradation and inhibited its transcriptional activity (**Figure 4.8A**).

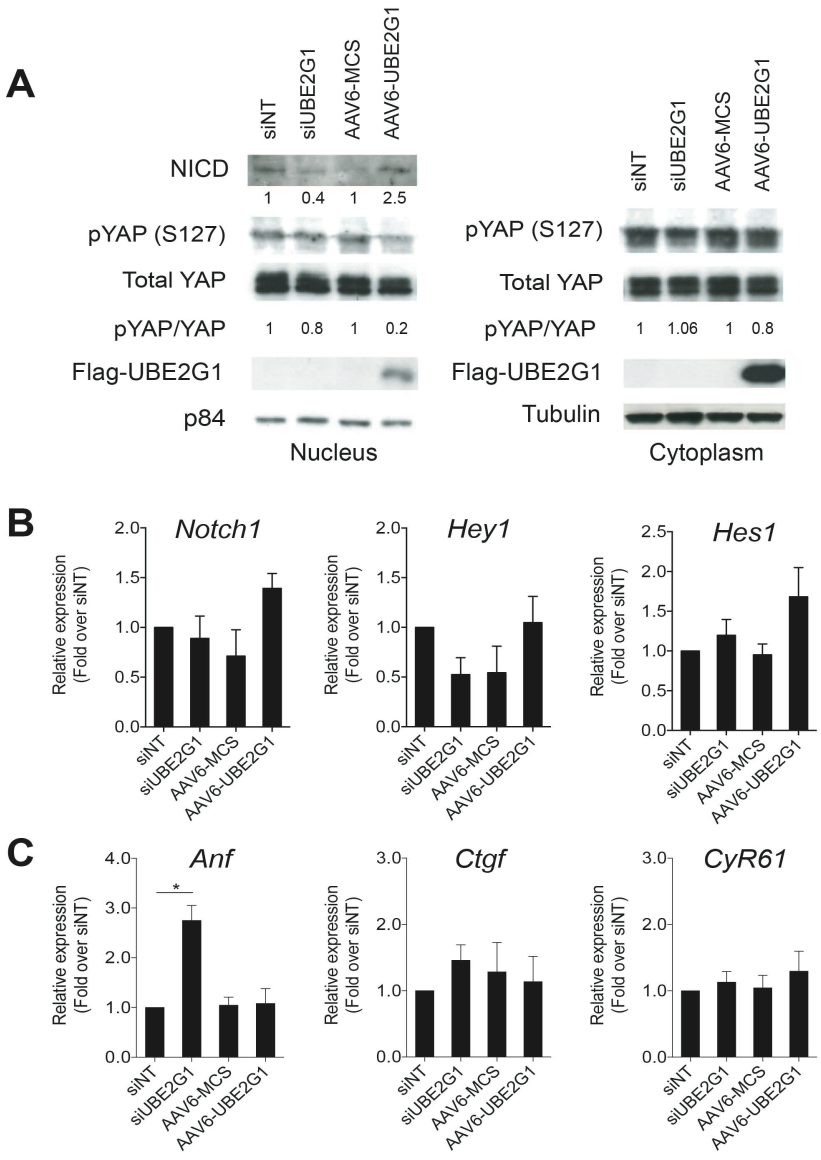

Figure 4.8: The interaction of UBE2G1 with Notch1 and Hippo pathways. A. Representative blots showing the protein levels of nuclear and cytoplasmic NICD,

phosphorylated YAP (Serine 127), and total YAP of treated samples. p84 and Tubulin served as controls of cellular fractionation. The numbers show blot quantification of siUBE2G1 normalized by siNT, and AAV6-UBE2G1 normalized by AAV6-MCS. **B.** Relative expression of *Notch1*, *Hey1,* and *Hes1* of indicated samples. Data are mean+s.e.m. **C.** Relative expression of *Ctgf*, *CyR61,* and *Anf* of indicated samples. Data are mean ± s.e.m (n = 3 independent experiments), *p<0.05, one-way ANOVA.

We further investigated these pathways by quantifying the expression of *Notch1* and its downstream genes (*Hey1* and *Hes1*) at the mRNA levels, and the same applied for YAP-responsive genes such as *Ctgf, CyR61,* and *Anf.* Consistent with the previous results, our data showed the reductions of *Notch1* and *Hey1* expression upon treatment with siUBE2G1 while the overexpression of UBE2G1 induced the mRNA expression of *Notch1*, *Hey1* and *Hes1* (**Figure 4.8B**). Intriguingly, we observed that siUBE2G1 treatment exerted a significant induction of *Anf* expression, a known hypertrophic marker (**Figure 4.8C**). This result was relevant to the previous data shown in panel **4.3**. No significant change was observed for *Ctgf* and *CyR61*, connected with Yap induced proliferation (**Figure 4.8C**).

In conclusion, our data show that UBE2G1 acts through numerous mechanisms and signalling pathways to mediate cardiomyocyte replication. However, the direct target(s) remains still unknown.

4.7 UBE2G1 overexpression increases cardiomyocyte replication *in vivo* and preserves cardiac function at an early time point after myocardial infarction

Our data showed that the upregulation of UBE2G1 increases proliferation *in vitro*. Hence, we asked whether AAV-UBE2G1 was able to enhance proliferation *in vivo*. To answer this question, we injected AAV9-UBE2G1 in neonatal mice and looked at myocyte proliferation after 7 days. AAV9-MCS and AAV9-miR-199 were used as empty control and positive control of proliferation, respectively. The AAVs were injected intraperitoneally in neonatal mice (postnatal day 0) at a MOI of $3x10^{11}$ (vg per animal). EdU

was injected at a dose of 30 mg/kg, every other day until sacrifice (**Figure 4.9A**). Frozen tissue was used to evaluate transgene overexpression, while paraffin-embedded tissue was used to assess cell proliferation.

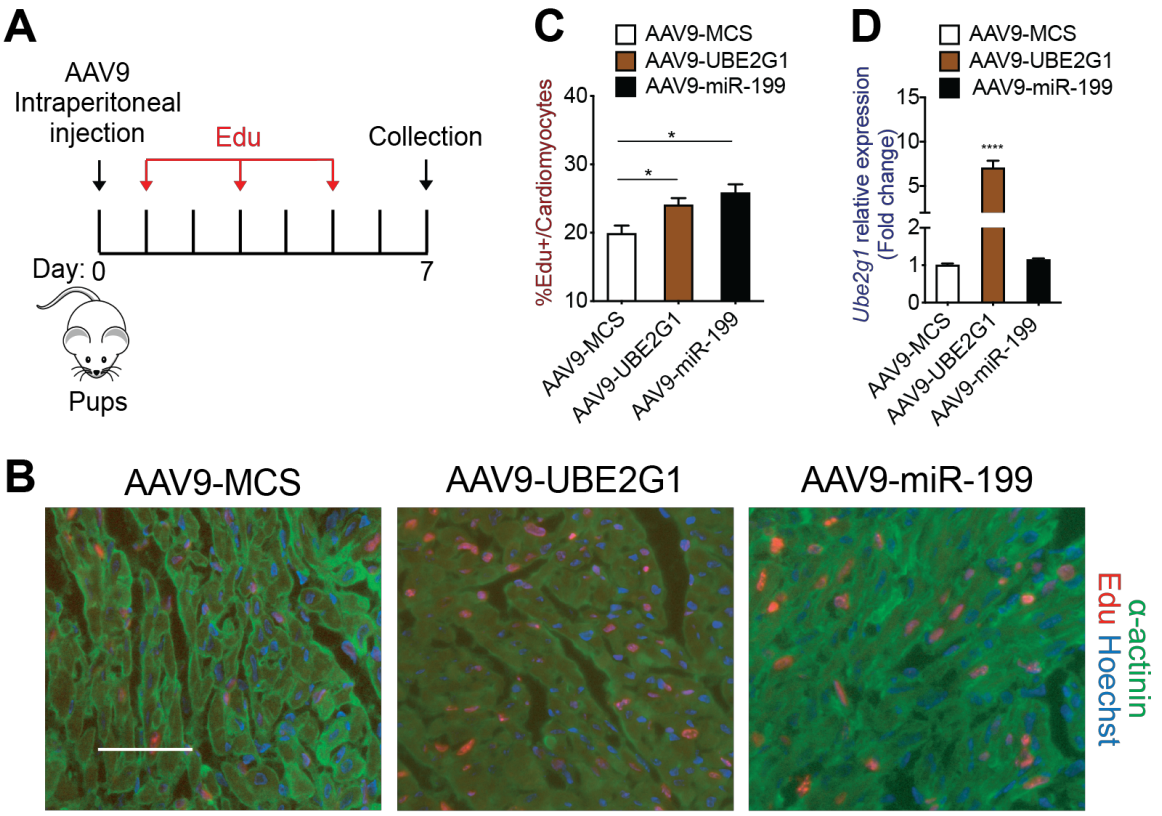

Figure 4.9: UBE2G1 overexpression increases cardiomyocyte replication *in vivo*. **A.** Experimental flowchart; each mouse was injected with $3x10^{11}$ viral particles and received EdU every other day before heart collection. **B.** Representative images of EdU-positive cardiomyocytes from the heart sections with the indicated treatments. Scale bar 50 μm. **C.** Percentage of cardiomyocyte proliferation at 7 days after injection. Data are from the quantification of over 2000 cardiomyocytes in each animal. Data are mean ± s.e.m (n ≥ 5 animals per condition), *p<0.05, t-test. **D.** UBE2G1 relative expression from the heart of treated mice; data are mean ± s.e.m (n = 3 independent experiments), *p<0.05, **p<0.01, t-test.

We observed a significant increase of EdU positive cardiomyocytes in the AAV9-UBE2G1 treated group compared with the control group (**Figure 4.9B and C**; the data show the counts of at least 2000 alpha-actinin positive cardiomyocytes per animal, from two independent experiments. Transgene overexpression by AAV9 was confirmed by qPCR (**Figure 4.9D**).

Then, we moved to adult mice to test the ability of UBE2G1 in preserving heart function after myocardial infarction (MI). Adult mice underwent permanent ligation of the left anterior descending coronary artery and were immediately injected with $3x10^{11}$ viral particles of AAV9-UBE2G1 or AAV9-MCS. Animals were monitored by echocardiography for two months. We measured the left ventricle ejection fraction (EF) and left ventricular anterior wall thickness (LVAWd) at 15, 30, and 60 days after MI (**Figure 4.10A**).

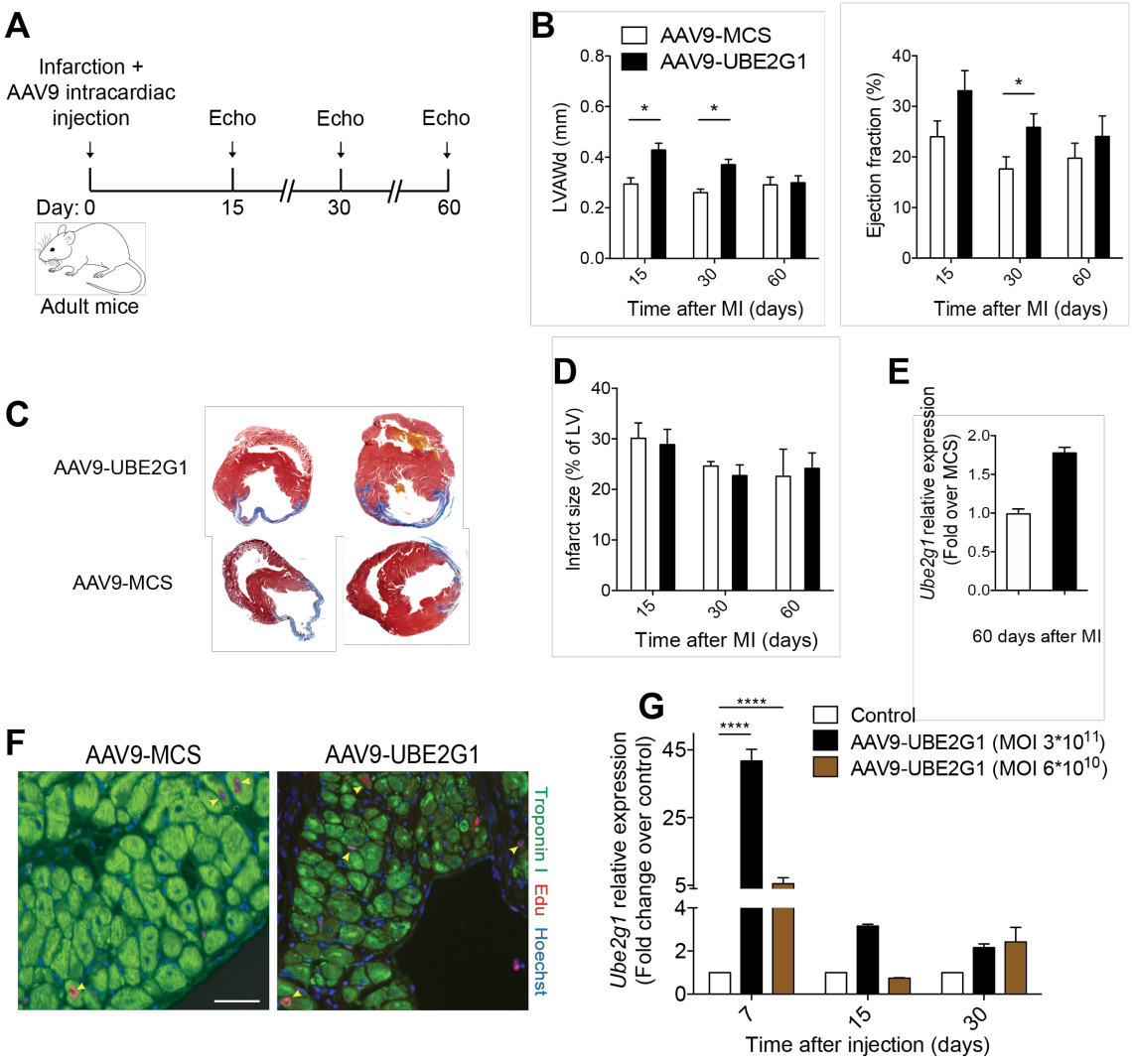

Figure 4.10: UBE2G1 overexpression preserves heart function at early time points after myocardial infarction. A. Experimental flowchart (Echo: echocardiography). **B.** Heart function at indicated time points (EF, Ejection Fraction; LVAWd, Left Ventricular Anterior Wall thickness). Data are mean ± s.e.m (n ≥ 10 animals per condition), *p<0.05, t-test; **C.** Representative images of the heart sections after Masson trichrome staining at 60 days after MI, fibrotic areas are stained in blue. **D.** Quantification of infarct size expressed as the percentage of left

ventricular volume. **E.** UBE2G1 relative expression from the heart of treated mice at 60 days after MI. **F.** Representative images of proliferating cardiomyocytes from the heart sections of treated mice. Scale bar 20 μm. **G.** UBE2G1 relative expression from the heart of treated mice normalized by control mice at 7, 15, and 30 days after injection.

The results showed significant preservation of LVAWd and EF in the treated group compared to control at 15 and 30 days after MI. However, we could not see a relevant difference between the two groups at 60 days (**Figure 4.10B**). After sacrifice, paraffin-embedded tissues were used to monitor tissue fibrosis by trichrome staining. We did not observe any difference in the infarct size between the two groups (**Figure 4.10C and D**). To better understand this unexpected result, we quantified the transgene overexpression at two months after injection. UBE2G1 expression was barely detected (**Figure 4.10E**). In parallel, EdU positive cardiomyocytes were almost undetectable at this late time point (**Figure 4.10F**).

Observing the low expression of UBE2G1, we wondered whether this was a possible explanation for the low preservation of cardiac muscle (no difference in infarct size). To answer this question, we performed a time-dose-dependent experiment in adult mice. CD1 animals received intracardiac injection with the MOI $3x10^{11,}$ or $6x10^{10}$ of AAV9-UBE2G1 and were sacrificed at 7, 15, and 30 days after injection to quantify UBE2G1 expression. On day 7, we observed a 40-fold increase in the UBE2G1 mRNA in mice injected with a higher dose of AAV9-UBE2G1 compared with control mice. Only 5-fold enrichment was detected in the mice injected with a lower dose of AAV9-UBE2G1 (**Figure 4.10G**). However, UBE2G1 expression started to decline dramatically in both groups at 15 days after injection (**Figure 4.10G**). Thus, this loss of UBE2G1 expression over time, possibly due to a toxic effect on the expressing cells, might partially explain why we could not observe significant preservation of cardiac function after MI.

5. DISCUSSION

In this work, we report the results of a high throughput screening, fluorescence microscopy-based, in neonatal mouse cardiomyocytes using a library of siRNAs against more than 600 target mRNAs coding for proteins in the ubiquitination to search for the factors involved in cardiomyocyte proliferation.

Ubiquitination plays a crucial role in proteostasis to ensure a continuous turnover of proteins, remove misfolded molecules, and recycle those no longer needed by the cells (Pagan et al. 2013). Loss of cellular protein homeostasis results typically in proteotoxic stress and accumulation of reactive oxygen species (ROS) (Dunlop et al. 2009). This condition is commonly associated with the pathogenesis of aging-related diseases such as neurodegenerative and cardiovascular disorders. In particular, cardiomyocytes have minimal regenerative capacity that lessens proteotoxic removal happening through cell division, and their high metabolic demand favours reactive oxygen species (ROS) accumulation (Hofmann et al. 2019). Targeting ubiquitination represents an unexploited therapeutic approach in heart diseases (Henning and Brundel 2017) and several studies have already supported this concept. For example, treatment with lactacystin (a proteasome inhibitor) prolonged the half-life of myosin heavy chain (Eble et al. 1999), or dysfunction of ubiquitination promoted cardiac hypertrophy and heart failure (Chen et al. 2005).

In this context, my research project was specifically aimed at identifying the factors in the ubiquitination system that function as positive regulators of cardiomyocyte proliferation. On the one hand, this can provide new insights into the mechanisms regulating the cardiomyocyte cell cycle while, on the other, it can lead to the identification of new possible targets for the development of regenerate therapies in the heart.

Some ubiquitination factors essential for cardiomyocyte proliferation were identified from our large scale screening, including E2 conjugating

enzymes, E3 ligases, and deubiquitinases. Among the top eleven hits of factors I found to act as crucial regulators of cardiomyocyte replication, we found the E3 ligase PHF5A. This identification was considered as a good marker of the screening efficacy since PHF5A depletion was already reported to reduce the self-renewal potency of embryonic stem cells (Strikoudis et al. 2016). Interestingly, most of the identified genes have not been associated with cardiomyocyte replication so far, strengthening the novelty of our study.

Since the knock-down of these genes hampered cell proliferation, we asked whether their overexpression, on the contrary, might enhance cardiac myocyte replication. To efficiently deliver the genes of interest into cardiomyocytes, we exploited the cardiac tropism and long-term expression of Adeno-Associated Viral (AAV) vectors, largely used for both *in vitro* and *in vivo* gene delivery in our laboratory (Lovric et al. 2012; Ruozi et al. 2015; Gabisonia et al. 2019). We observed that the overexpression of a few of the selected genes including DCUN1D3, SOCS5 and UBE2G1 could induce cardiomyocyte proliferation *in vitro.* Some E3 ligases, such as MuRF1, FBXO32, or MDM2, were reported to protect the heart against either oxidative stress or ischaemia-reperfusion injury (Brown et al. 2017b; Dadson et al. 2017). Our results added DCUN1D3 and SOCS5 to the group of E3 ligases potentially interesting to provide a beneficial effect to the heart. The most interesting factor from this validation assay was the E2 conjugating enzyme UBE2G1, which exhibited the strongest pro-proliferative effect and thus was selected for further investigation. Much of E2 functions remain to be discovered in the heart, UBC9 is a rare E2 enzyme that was identified to regulate proteostasis in cardiomyocytes (Gupta et al. 2014), while the factor that associate to myocyte replication mostly remain still elusive.

The human UBE2G1 gene is located on chromosome 17p13.3 and encodes a highly conserved, but poorly characterised, E2 conjugating enzyme, which was initially described in a medulloblastoma study (Cvekl Jr

et al. 2004). This enzyme is known to synthesise K-48 polyubiquitin without E3 ligase through a still poorly understood mechanism, and its acidic loop plays an essential role in this process (Choi et al. 2015). The UBE2G1 protein possesses a single catalytic domain with a crucial cysteine residue in the active site. The binding of modified ubiquitin to the backside of UBE2G1 was reported to interfere with the formation of thioester-linked of cysteine active site and the E1 enzyme (Garg et al. 2020). However, the mechanism to transfer the ubiquitin chain to the target remains elusive. In order to understand whether the pro-proliferative effect of UBE2G1 overexpression on cardiomyocytes was due to its catalytic activity, we substituted the cysteine residue to alanine in the catalytic loop, which had the effect of abolishing completely the pro-proliferative effect of this factor. Our data complement the previous report by Choi and colleagues showing that the substitution of the active cysteine by a serine was not significantly affecting K48-polyubiquitin chain synthesis (Choi et al. 2015), whereas the exchange between cysteine and alanine residue of UBE2G1 inhibited its activity. The exchange of cysteine to serine residue is a conservative replacement, while cysteine replaced by alanine impacts vigorously the intramolecular affinity of protein domains, possibly affecting the proper folding of the protein (Hizi et al. 1992). This explanation is consistent with the lack of any pro-proliferative effect by the mutant UBE2G1-CA protein in cardiac myocytes. Still, we do not exclude the possibility that the overexpression of UBE2G1-CA could compete with the endogenous protein, acting as a dominant-negative partner, therefore suppressing the downstream signalling and the consequent biological effect.

We showed that UBE2G1 is essential for cardiomyocyte cell cycle entry, as the depletion of this factor arrests the cells in the G1 phase and prevents cell cycle progression by FACS analysis. We reinforced this observation by presenting evidence that UBE2G1 inhibition abolished the pro-proliferative effects of miRNAs (miR-199a-3p, miR-590-3p, miR-302c, miR-373, and miR-1825) previously demonstrated to induce myocyte proliferation (Eulalio et al. 2012). Our data are consistent with the findings that UBE2G family

members, namely UBE2G1 and UBE2G2, control the degradation and ubiquitylation of the replication licensing factor CDT1 and Cyclin-dependent kinase (Cdk) inhibitor p21, respectively. Origin licensing is the first committed step of DNA replication and its control is intimately coordinated with the mechanisms that govern cell cycle progression. In mammalian cells, small changes in CDT1 leads to catastrophic consequences of genome stability (Arentson et al. 2002; Tatsumi et al. 2006; Blow and Gillespie 2008), suggesting that CDT1 regulation is strictly necessary. Moreover, the critical aspect of re-replication control in metazoans is ubiquitin-mediated, and CDT1 degradation during S phase is known to be controlled by different E3 ubiquitin ligase complexes (Abbas and Dutta 2011; Havens and Walter 2011; Petroski and Deshaies 2005).

As mentioned above, UBE2G1 is associated with CDT1 stability for modulating cell cycle progression, thus a logical step in our experiments was to demonstrate a direct (or indirect) interaction between UBE2G1 and the $CRL1^{Skp2}$ complex. Indeed, this complex controls the degradation of CDT1, thus affecting the G1/S entry of myocytes in the cell cycle. In cultured cardiomyocytes with overexpression of UBE2G1 protein, we could not detect direct interaction of UBE2G1 and CDT1 protein by co-immunoprecipitation, while we were able to confirm the CDT1 and CUL1 interaction, as it was previously described by Li and colleagues (Li et al. 2003). To explain these results, we have to consider that the identification of E2 conjugating enzyme partners is a major obstacle in studying E2 functions, as they are known to interact with multiple E3 ligases through a relatively weak and transient interaction that leads to a speedy degradation of their substrates (Polge et al. 2015). Thus, it might be possible that the detection of a direct interaction between UBE2G1 and the CDT1 degradation complex might have escaped our co-immunoprecipitation studies. Further investigations are needed to unravel the network of proteins responsible for the biological effect that we detect upon overexpression of UBE2G1. In recent work, an E2 thioester approach (E2~dID) has been developed to identify the substrates of UBE2C and its

partner E3 ligase (Bakos et al. 2018). This strategy can be pursued in our case, as well, by the creation of a mutant UBE2G1 able to covalently and permanently bind the interacting E3 enzyme without promoting the degradation of its substrates. In addition, through an immunoprecipitation assay, we also excluded the possible interaction between UBE2G1 and any of the E3 ligases identified in our screening as potential modulators of cell cycle progression. Another possible explanation for the apparent lack of direct interaction between UBE2G1 and an E3 ligase might reside on the unique feature of UBE2G1 and UBE2R1, which can synthesize K-48 polyubiquitins without an E3 ligase (Choi et al. 2015). And the absence of evidence for a direct molecular interaction between UBE2G1 and any E3 known ligase supports this possibility.

UBE2G1 is involved in cell cycle regulation by controlling CDT1 and p21 protein levels in response to UV irradiation (Shibata et al. 2011). Following this observation, we expanded the panel of cell cycle factors that might be affected by UBE2G1. We noticed that, upon the silencing of UBE2G1, the expression levels of cyclin D1 and cyclin E, the two factors that are required at the G1/S checkpoint (Resnitzky et al. 1994), were markedly reduced. Conversely, p27 expression was induced significantly at both transcriptional and protein levels. This result reinforces our hypothesis that UBE2G1 participates in the selective ubiquitylation of substrates involved in cell cycle regulation.

We further investigated the signalling cascades modulated by UBE2G1 protein (upon overexpression and silencing) in cultured cardiomyocytes by evaluating the phosphorylation profile of 43 common kinases expressed in this cell type. We found that, in UBE2G1 overexpression condition, the GSK-3β, STAT3, and ERK1/2 pathways were affected. In particular, the upregulation of UBE2G1 induced inactivating phosphorylation of GSK-3β, a known inhibitor of cardiomyocyte replication (Lal et al. 2015; Singh et al. 2018; Tong et al. 2002). Consistently, ERK1/2 activation is enhanced to mediate cell proliferation (Lorenz et al. 2009; Wang 2007). In the case of

STAT3, UBE2G1 protein functions through a non-canonical mechanism which has been involved in cell proliferation, survival, and the enhancement of transcriptional activity (Hazan-Halevy et al. 2010; Ouedraogo et al. 2016). Together, these evidence support the pleiotropy of targets regulated by the UBE2G1 enzyme, strengthening the indication that this factor acts on the cardiomyocyte cell cycle by modulating multiple targets and thus tuning a coordinated signalling network toward a proliferative response.

In this view, we focused our attention on the biochemical cascades that we and others have demonstrated to drive cardiomyocyte proliferation, namely the Notch1 and Hippo pathways (Collesi et al. 2008; Torrini et al. 2019). The ubiquitination cascade tightly controls the amplitude of both pathways in different manners (Kim and Jho 2018; Moretti and Brou 2013). For example, the E3 ligase ITCH targets and destabilizes the LATS1 kinase to suppress the downstream signalling, while the Notch1 intracellular domain (NICD) is the known substrate of the nuclear E3 ligase FBXW7, which ensures its fast recycling on target promoters and rapid degradation in physiological circumstances (Close et al. 2019; Salah et al. 2011). Moreover, recent data from our laboratory demonstrated that activated Notch1 half-life is extended by acetylation, a post-translational modification that competes with the ubiquitylation to enhance its transcriptional activity and sustain cardiomyocyte proliferation (Collesi et al. 2018). Of note, an E2 conjugating enzyme involved in regulating these pathways has not been identified yet.

Based on the above considerations, we asked whether UBE2G1 levels could affect the activity of these pathways. Our results showed that the silencing of UBE2G1 was paralleled by reduced amounts of NICD in transfected cells, while its overexpression increased the nuclear NICD protein level, subsequently sustaining the proliferative capability of cultured cardiac myocytes. These data were supported by transcriptional upregulation of Notch1 known target genes, such as *Hes1* and *Hey1* when UBE2G1 was overexpressed. In a similar experiment, we noticed an

interesting connection between UBE2G1 and YAP activity. The fractionation of transduced cardiomyocytes did not show a significant change in the nuclear levels of YAP affected by the shift in UBE2G1 levels. Consistently, we could not detect any significant difference in the expression of *Ctgf* and *CyR61*, two known YAP-responsive genes positively involved in cell proliferation. Conversely, in the case of UBE2G1 silencing, we observed a significant increase of atrial natriuretic factor (ANF), a hypertrophic marker. Our results are consistent with a previous report by Yang and colleagues, showing that the constitutive expression of YAP triggered cardiac hypertrophy and inhibited cell death both *in vitro* and *in vivo* through miR-206 overexpression. Similar to our results, these authors presented a significantly increased of *Anf* mRNA by miR-206 upregulation and YAP activation, which regulated the hypertrophy and survival of cardiomyocytes but did not exert any significant pro-proliferative effect (Yang et al. 2015). These data are relevant to our observation on the hypertrophic phenotype of cardiac myocytes when we treated cells with siUBE2G1 and miRNAs. We can argue that, in these conditions, UBE2G1 suppression impacts on the cytoskeletal structure of cardiomyocytes, which needs to be reshaped to a hypertrophic response. A similar observation of UBE2G1 modification affecting cardiac myocyte structure was reported by Haghikia and colleagues by showing that its silencing resulted in the loss of several sarcomeric proteins, including cardiac myosin heavy chain and troponin although the induction of hypertrophy was not reported (Haghikia et al. 2010).

To better clarify the impact of UBE2G1 overexpression on cardiomyocyte proliferation, we first tested the effect of this factor *in vivo* by evaluating the proliferative capacity of neonatal mouse hearts after the injection of AAV9-UBE2G1, followed by EdU administration to measure the cell turnover. At seven days after the delivery of the AAV vector, we observed a significant increase in EdU incorporation, consistent with our *in vitro* data. We then wondered whether UBE2G1 overexpression could exert a therapeutic effect after myocardial infarction in adult mice. We found that a single

intracardially administration of AAV9-UBE2G1 significantly preserved the heart function of adult mice at 15 and 30 days after myocardial infarction, while the beneficial effect was not significant at 60 days. We attribute this lack of effect at longer times to a draft decrease of the transgene expression in the heart of injected mice. The loss of transgene expression *in vivo* is not a sporadic event with AAV vectors, since it was reported in several clinical trials to deliver the factor IX to hemophilic patients. In these cases, the drop of factor IX levels was a time-dependent event suggesting the transduced hepatocytes were removed gradually by the immune system (Manno et al. 2006; Nathwani et al. 2014). Although this event was barely mentioned in rodent, it is a feasible explanation for the drop of UBE2G1 expression after myocardial infarction, as the boost of the inflammation might provoke cell-mediated immunity targeting antigens of the AAV capsid, followed by the removal of transduced cells and decreased the beneficial effect (Frangogiannis 2014; Santos-Zas et al. 2019). Apart from preventive immunosuppression, some strategies can be conceived to avoid immune reactivity against AAV vectors. A widely investigated approach involves the modification of capsid immunogenic epitopes by random mutagenesis, also by including epitopes specifically activating suppressive T regulatory cells (Li et al. 2012). Another possibility harnesses the use of empty capsids as decoys to adsorb anti-AAV antibodies, although increasing the viral particle load may increase the cytotoxic T lymphocyte response (Mingozzi and High 2013). Notably, a recent study on chondrocyte cell line C28/I2 reported that circulating mRNA-UBE2G1 affected the expression levels of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α (Chen et al. 2020). Therefore, further studies appear required to understand the possible relation of UBE2G1 and the immune system in order to prevent the loss of AAV9-UBE2G1 transduced cells due to the clearance by the immune response.

As mentioned earlier, ubiquitination plays irreplaceable roles in protein quality control as a priming system to dispose the proteins with abnormal

modifications (Pagan et al. 2013). Post-translational modifications by this system are faster and more efficient in response to environmental changes as it does not require the synthesis of molecules and can be reversed when the stimulus vanishes (Ashida et al. 2014). The versatility of ubiquitination makes it an attractive target for different fields of biomedical research although the lack of functional studies and fragmentary literature have restricted its applicative translation (Wertz and Wang 2019).

Herein, we report for the first time the roles of the E2 conjugating enzyme UBE2G1 on cardiomyocyte proliferation both *in vitro* and *in vivo.* Our data reveal that this factor is capable of enhancing the replication of cardiac myocytes by modulating the levels of different cell cycle regulators. We also demonstrate a possible interaction of UBE2G1 with some pivotal signalling cascades driving cell proliferation, highlighting the multifaceted effect of this E2 conjugating enzyme on various cell functions.

Further investigations require the identification of the E3 ligase partners (if any), of UBE2G1 in the heart and its ultimate substrates, in order to shed light on the actual molecular mechanisms that mediate the progression of cell cycle and enhance cardiomyocyte proliferation by this factor. Moreover, several experiments can be performed to better clarify the biological function of UBE2G1. These include: (i) to identify the impact of UBE2G1 on p27 stability as its silencing increased the half-life of this protein, thus the E3 ligase complexes that participate in this process can be assessed for the interaction; (ii) to validate the silencing effect of UBE2G1 on cardiomyocyte replication and hypertrophy *in vivo*; an AAV9-shUBE2G1 has already been generated for this experiment; (iii) to investigate the possible relation of UBE2G1 with different complexes that involve in DNA origin licensing beside CDT1, such as MCM, ORC and CDC.

6. PUBLISHED PAPERS

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